Original antigenic sin in Japanese encephalitis vaccination

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Filippo Tatullo

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List of abbreviations

AA	Amino acid
ADCC	Antibody dependent cellular cytotoxicity
ADE	Antibody dependent enhancement
ADEM	Acute disseminated encephalomyelitis
AFRIMS	Armed Forces Research Institute of Medical Sciences
APC	Antigen presenting cell
BBB	Blood brain barrier
BCIP/NBT	5-bromo-4-chloro-3-indolyl-phosphate/ nitro blue tetrazolium
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CPE	Cytopathic effect
CRI	Cross-reactivity index
CTL	Cytotoxic T lymphocytes
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DENV	Dengue virus
DHF	Dengue haemorrhagic fever
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ds	Double strand
E	Envelope
ER	Endoplasmic reticulum
FCS	Fetal calf serum

FL	Fusion loop
GMT	Geometric mean titre
HBEC	Human bronchial epithelial cells
HBSS	Hanks' balanced salt solution
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocytes antigen
HPV	Human papillomavirus
HRP	Horseradish peroxidase
ICS	Intracellular Cytokine Staining
IFN	Interferon
IFNγ	Interferon-γ
IL	Interleukin
IRF	Interferon-regulatory factor
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
KO	Knock out
LAJV	Live attenuated Japanese encephalitis vaccine
LMP	Low melting point
mAb	Monoclonal antibody
MDA-5	Melanoma differentiation-associated protein 5
MEM	Minimum essential medium
MHC	Major histocompatibility complex

MOI	Multiplicity of infection
MP	Mini pool
MVEV	Murray Valley encephalitis virus
NAb	Neutralising antibody
NIMHANS	National Institute of Mental Health and Neurosciences
OAS	Original antigenic sin
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween 20
PCR	Polymerase chain reaction
PFU	Plaque forming unit
PMA	Phorbol 12-myristate 13-acetate
prM	Pre-membrane
PRNT	Plaque reduction neutralisation test
PRR	Pattern recognition receptors
P/S	Penicillin/streptomycin
RDRP	RNA dependent RNA polymerase
RIG-I	Retinoic-acid inducible gene I
RLR	Retinoic acid-inducible gene-I-like receptors
RNA	Ribonucleic acid
rpm	Revolution per minute
RPMI	Roswell Park Memorial Institute

SCR	Seroconversion rate
SFC	Spot forming cell
ss	Single strand
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
SVP	Subviral particles
UTR	Untranslated region
TCL	T cell line
TCR	T cell receptor
TDV	Tetravalent dengue vaccine
TGN	Trans-Golgi network
Th	T helper
TLR	Toll like receptor
TM	Transmembrane
TMB	3,3′,5,5′-tetramethylbenzidine
TNFα	Tumor necrosis factor-α
Treg	Regulatory T
WNV	West Nile virus
WT	Wild type
YFV	Yellow fever virus
ZIKV	Zika virus

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Abstract

Japanese encephalitis (JE) virus (JEV) is the main cause of viral encephalitis in South and South-East Asia. JEV is a member of the *flavivirus* Genus (Family *Flaviviridae*) and it is transmitted by mosquitoes. A live attenuated vaccine is used in many endemic countries. Dengue virus (DENV) is another flavivirus, closely related to JEV, also transmitted by mosquitoes, which is endemic in all tropical and subtropical countries, including India.

This study aimed to analyse the antibody and T cell responses to the live attenuated JE vaccine (LAJV) and their cross-reactivity with the 4 DENV serotypes in adult Indians.

Participants were vaccinated with the LAJV and peripheral blood mononuclear cell and serum samples were collected before and after vaccination. Antibody responses specific to JEV and DENV were studied by plaque reduction neutralisation test and ELISA whereas T cell responses were measured by interferon γ - ELISpot and intracellular cytokine staining.

Neutralising antibody (NAb) response to the LAJV was observed in 3 out of 16 volunteers (18.75%) with a peak reciprocal geometric mean titre of 20 at 4 weeks after vaccination. Ten volunteers (62.5%) were DENV exposed at baseline, as assessed by IgG ELISA. Interestingly, original antigenic sin (OAS) was observed in approximately 30% of DENV exposed individuals, as shown by the fact that they mounted a NAb response to DENV rather than JEV following vaccination. OAS was observed in responders as well as non responders. In addition, data suggested DENV NAb at baseline could increase immunogenicity of the vaccine.

Previous work performed by others on this cohort showed that T cell responses, which peaked at 2 weeks following vaccination, were identified in most volunteers by ELISpot. Furthermore, T cell responses specific to JEV were detected following vaccination even in volunteers without antibody response.

Five T cell epitopes were identified after vaccination and 4 of them showed cross-reactivity with one or more DENV variants. Three of them were also detected before vaccination. T cell data indicated that the vaccine was able to induce *de novo* DENV cross-reactive as well as JEV specific T cell responses. Finally, previous cross-reactive DENV responses did not inhibit *de novo* T cell responses.

Introduction

Japanese encephalitis

Japanese encephalitis (JE) is a disease caused by Japanese encephalitis virus (JEV). Symptomatic infection occurs in 0.1-1% of people infected, with the ratio of asymptomatic to symptomatic infection varying with age[1,2]. JE can present a different range of clinical severity going from a fatal meningoencephalomyelitis to milder forms like aseptic meningitis or flu like illness[3].

Following infection, after an incubation period of 5-15 days (median of 8.4 days), symptoms start with a non-specific febrile illness[4]. Other clinical features include coryza, diarrhoea, rigors, vomiting and headache[5]. Disease may progress successively with altered consciousness, focal neurologic deficits, generalized weakness, movement disorders and convulsion. Seizures are more common in children (observed in 85% of cases) than adults (reported in roughly 10% of adult JE patients)[5]. Older children and adults may also present abnormal mental behaviour that can potentially mislead towards a diagnosis of mental illness[6].

The clinical presentation of a patient with JE includes: a dull, flat mask-like facies, unblinking eyes, tremor, hypertonia and rigor. Other extrapyramidal neurological features include head nodding, pill rolling movements, lip smacking and facial grimacing. Multiple seizures are associated with poorer prognosis[7].

A poliomyelitis like acute flaccid paralysis has been observed in some JE patients[8]. This condition can affect one or more limbs although weakness is more prominent in the lower limbs with asymmetrical wasting. Nerve conduction studies and electromyographic test demonstrated anterior horn cell damage[9].

JE has a mortality rate of approximately 18%. Around 44% among survivors are left with permanent neurological sequelae with important finance and social consequences[10].

There is no specific treatment for JE, and management is based on supportive care. Vaccination and, in theory, precautions to avoid mosquito bites, can prevent the disease.

History

Outbreaks of encephalitis were recognised in Japan from 1871, with recurrent epidemics which occurred during summer. The disease was named "Japanese B encephalitis" to distinguish it from von Economo's encephalitis, known as type A; the "B" has since fallen out of use.

In 1924 a filterable agent from a fatal case of JE was shown to induce encephalitis in monkeys. In 1935, the prototype Nakayama strain of JEV was isolated from the brain of a fatal case[5].

Virology

JEV belongs to the genus *Flavivirus*, family *Flaviviridae*, which includes 3 other genera: *Hepacivirus, Pegivirus* and *Pestivirus*. The genus *Flavivirus* includes over 70 viruses some of which are transmitted by vectors such as ticks and mosquitoes. The genus is further divided in serogroups such as the dengue serogroup which includes the 4 dengue virus (DENV) serotypes, the yellow fever serogroup which includes yellow fever virus among them, the spondweni group which includes Zika virus (ZIKV), and the JE serogroup which includes JEV, West Nile virus (WNV) and Murray Valley encephalitis virus (MVEV). The structure and genomic organization is conserved among all flaviviruses. The mature virion has a diameter of approximately 50 nm and contains a single positive strand ribonucleic acid (RNA) genome approximately 11kb in size. The 5′ end is capped; the 3′ end lacks a poly-A tail. The genome is organized in a long single open reading frame flanked by 5′ and 3′ untranslated regions (UTR), from which a single polyprotein is made before being subsequently cleaved by host and viral proteases. There are 10 mature viral proteins after processing, which include 3 structural (core (capsid), membrane and envelope (E)) and 7 non-structural proteins (NS1, NS2a and b, NS3, NS4a and b and NS5)[11]. The 5′ and 3′ non coding regions produce sub-genomic RNAs that are involved in interfering with the innate immune response, such as production of type I interferons[12].

The core protein is a small protein of approximately 114 amino acids (AA). The core is characterized by basic AA clusters which give it RNA binding properties. Although being part of the structure of the virion, core protein was also found in nuclei of the cells. Blocking transit of the core protein to the nucleolus decreased viral replication in mammal cells and neuroinvasiveness in mice[13].

The membrane protein is another structural protein, which is produced as pre-membrane (prM) protein. The main role of the prM is to regulate virus assembly. In particular, it works as chaperone helping the E protein in folding correctly[14,15]. The pr fragment has a crucial function during the process of virus maturation (described below).

The last structural protein is the E protein which is a glycosylated protein of approximately 500 AA. In the mature virion, E is organized in antiparallel dimers. Each virion contains 180 E proteins organized in 90 dimers which are arranged in a herringbone fashion with icosahedral T = 3 symmetry (figure 1). The E protein has 3 domains: EDI, EDII and EDIII. EDI stabilizes the overall orientation of the protein and it works like a bridge hinge among the other two domains. EDII is responsible for the antiparallel homodimerization and contains the highly conserved hydrophobic fusion loop (FL) which mediates virus entry by fusing the E with the membrane of the endosome. Finally, EDIII is involved in receptor binding[16].

E is an extremely dynamic protein that goes through structural rearrangements triggered by environmental pH. The appearance of the mature virion at neutral pH is smooth; however, during the virus entry, in the acidic pH of the endosome the E protein undergoes dramatic rearrangement[16]. The EDII hinges outward exposing the FL that inserts into the host cell membrane of the endosome. This promotes formation of 60 E trimers that move to create trimer contacts bending the membranes and promoting the formation of the fusion pore (figure 2)[17,18].

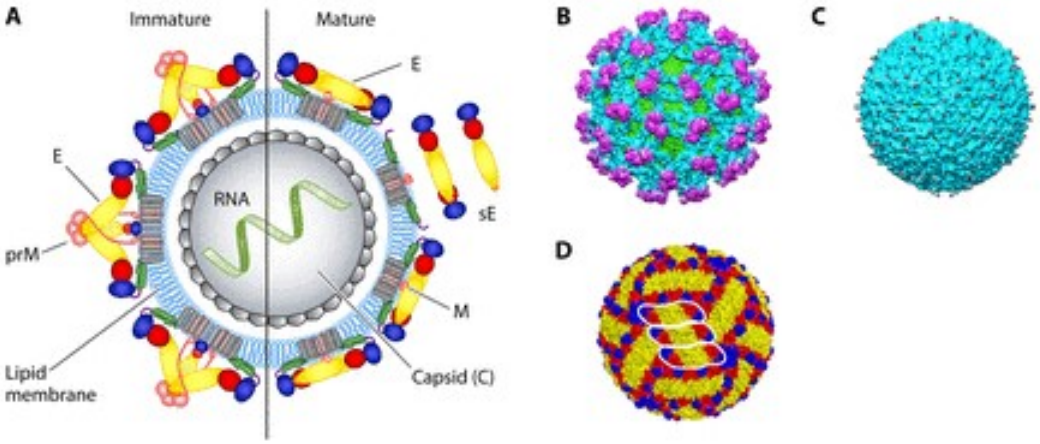


Figure 1. Schematics and structures of flavivirus particles.

Representation of immature (left) and mature (right) virions (A). Cryogenic electron microscopy structures of immature and mature DENV 1 (B and C). Surface representations of the herringbone arrangement of E dimers at the surface of mature dengue. EDI is coloured in red, whereas EDII in yellow and EDIII in blue (D). Figure reprinted from reference[19].

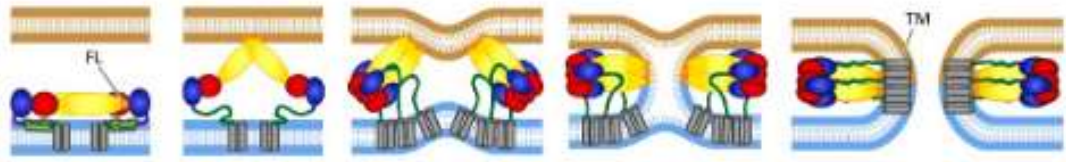


Figure 2. Schematic representation of flavivirus membrane fusion.

The acidic pH in the endosome promote the formation of E trimers which expose the FL. Further structural changes lead to the relocation of EDIII and the formation of a trimer in a hairpin-like structure in which the FL and the transmembrane (TM) regions are juxtaposed. Subsequently, the E trimers move to create trimer contacts providing the energy for membrane fusion. Figure reprinted from reference[19].

NS1 is a highly conserved glycosylated protein produced as a dimer, which is associated with endoplasmic reticulum (ER) and plasma membrane, and as a hexamer, which is secreted[20]. NS1 has been shown to co-localize with the replication complex and be crucial for viral replication[20]. DENV NS1 has been also implicated in the pathogenesis of severe dengue (described below). The JEV serogroup was shown to produce another version of NS1, together with the canonical version, called NS1′.

NS1′ is produced following a -1 ribosomal frameshift event due to a slippery heptanucleotide (YCCUUUU) and an RNA pseudoknot structure towards the 3′ end of the NS1 gene[20]. The final result is an NS1 protein with 52 extra AA that is involved in increased neuroinvasiveness[21].

NS2a is a small hydrophobic protein of approximately 231 AA which is associated with the ER membrane. It has been shown to strongly bind the viral RNA in the UTR region and be involved in viral genome replication in WNV[22,23]. Studies from WNV and DENV described also important roles for NS2a in virus assembly[24,25]. Finally, it has been implicated in interfering with the host immune response. NS2a of JEV is able to block the downstream effects of protein kinase R activation by inhibiting eIFα phosphorylation[26]. In WNV, a single mutation (A30P) of NS2a resulted in a mutant with attenuated neurovirulence and neuroinvasiveness *in vitro* and *in vivo*. The mutation induced expression of type I interferons indicating a role for NS2a in blocking the host antiviral immune response[27].

NS2b is a small membrane protein of approximately 130 AA. It works as a cofactor for NS3 forming a complex that acts as a serine protease. NS2b not only helps NS3 to fold correctly but it is also actively part of the substrate binding site of NS3 making direct contact with the substrate and therefore participating directly in the catalytic activity of NS3[28]. Although, the cytoplasmic portion of NS2b is sufficient to support the protease activity of NS3, the transmembrane domains are involved in other functions such as virion assembly and viral RNA replication[29,30].

NS3 is the second largest viral protein and consists of two domains which have two different functions[31]. The N terminus has a serine protease activity (in combination with NS2b) whereas the C terminus has nucleoside triphosphatase/ RNA helicase/ RNA triphosphatase functions[32]. This protease is essential for viral replication as it is involved in the cleavage of some viral proteins. Aguirre *et al.* described a role for DENV NS2b/NS3 complex in antagonising the antiviral response by cleaving the human stimulator of interferon genes (STING) protein[33]. STING is an adaptor protein that is localized in the ER and that, following activation, is able to promote activation of the transcription factors interferon-regulatory factor (IRF) 3/7 and NF-κB and therefore secretion of type I interferons and pro-inflammatory cytokines[34]. More recently, the motif RxEP present in NS3 protease domain of DENV was described to

be involved in ablating innate immune response blocking the translocation of retinoic acid-inducible gene I (RIG-I) from the cytosol to mitochondria. A similar motif (RLDP) was shown to work in a similar way also for WNV[35].

The helicase domain can recognise double and single strand (ds and ss) RNA and unwind it through the energy obtained by the nucleoside-triphosphatase activity[36]. Furthermore, an adenosine triphosphate independent RNA annealing function has also been described[37]. Finally, the RNA triphosphatase activity is considered to be the first step for the formation of the cap at the 5′ tail of the viral RNA[38].

NS4a is a small highly hydrophobic protein which works together with NS4b in anchoring the viral replication complex to the intracellular membrane. It consists of a hydrophilic N terminus which is found in the cytoplasm, a body of 2 hydrophobic transmembrane domains and a C terminal domain called 2K (because of its molecular weight of 2 kilodalton) that works as a signal sequence for NS4a translocation into the ER. Following translocation of NS4a in the ER the 2K fragment is cleaved by NS2b/NS3[39]. NS4a has also been implicated in activating the unfolded protein response, blocking type I interferons signalling[40] and inducing autophagy to prevent cell death[41], all effects which promote viral replication.

NS4b is also another hydrophobic protein of 27 kilodalton. DENV NS4b inhibits the type I interferons response whereas WNV NS4b is able to induce the unfolded protein response and blocks stress granules formation[40,42,43].

NS5 is the biggest and most conserved viral protein with approximately 900 AA. The protein consists of two domains: the N terminus which has methyl transferase activity and the C terminal which functions as a RNA dependent RNA polymerase (RDRP). These two domains are connected by a network of hydrophobic residues which, despite not being involved in the catalytic activities of the two domains, are highly conserved among all flaviviruses[44]. Another important function of NS5 is its ability to inhibit the host immune response. Despite NS5 is the most conserved protein, different mechanisms of immune interference have been described among different flaviviruses. JEV NS5 antagonises the host immune response in different ways: 1) it is able to block tyrosine phosphorylation of tyrosine kinase 2 and signal transducer and activator of transcription (STAT) 1 and the nuclear translocation of STAT1 by downregulating calreticulin and increasing the activity of calneurin[45,46], 2) it blocks

two nuclear transport proteins, KPNA3 and KPNA4, which are involved in the nuclear translocation of IRF3 and p65 (a subunit of NF-κB) which regulate the transcription of interferon β[47]. In contrast, WNV NS5 suppresses the expression of the interferon α receptor subunit 1 and therefore blocks antiviral signalling of type I interferons[48,49]. DENV and ZIKV NS5 antagonise type I interferons signalling by inducing STAT2 degradation by the proteasome[50–52].

Flavivirus life cycle

The first step in the flavivirus infectious cycle is to bind a receptor and enter the cell. Several proteins have been identified as putative receptors for the virus entry for different flaviviruses. Dendritic-cell-specific ICAM-grabbing non-integrin (DC-SIGN), the mannose receptor and CD14 were identified as receptors for DENV[53–55]. Heat shock protein 70, glucose-regulated protein 78 and heat shock protein 90β have been suggested as putative receptors for JEV[56–58]. Following virus binding to an entry receptor, the virus is internalized through clathrin coated pits and delivered to early endosomes which mature into late endosomes[59]. The low pH in this late endosome triggers conformational changes in the E protein which re-assemble as a trimer exposing the FL at the tip of the E trimer. The FL interacts with membrane of the endosome and EDIII folds back bending the membranes and promoting fusion of the viral and endosome membrane (figure 2)[60]. The capsid is introduced into the cytosol of the cell through the resultant pore. The released positive strand RNA is translated to produce a single polyprotein which is then cleaved by viral and host proteases. The viral genome is subsequently converted into negative sense RNA by the viral NS5 protein and works as template to generate further copies of the viral genome. Virus assembly occurs on the surface of the ER with the viral genome which is packed by the capsid protein present on the cytoplasmic side of the ER and the prM and E that assemble within the lumen of the ER. Subsequently, the viral particles move towards the Golgi and trans-Golgi network (TGN) to finally bud out through by exocytosis. The pr fragment, localized on top of the E protein, protects the E from premature fusion with the host membrane during the maturation process through the Golgi and TGN[61]. The low pH in the TGN promotes a structural rearrangement of the virion that exposes the prM to the cleavage of the host protease furin which cleaves the pr fragment from the parent prM protein[62]. Despite being cut, the pr fragment remains associated with the E protein and only detaches from it when the virion buds out of

the cell and encounters a neutral pH environment[63]. At this point, there is a conformational change leading the E protein to dimerise and become arranged horizontally along the viral surface, protecting the FL underneath EDIII of the adjacent E protein. The cleavage of the pr fragment is fundamental for the production of mature infectious viral particles. However, this process is not efficient and immature or partially mature virus particles are produced during viral replication[64,65]. These immature or partially mature viral particles are relevant especially for DENV as they can become infectious through a process of antibody dependent enhancement (ADE) (described below). Finally, subviral particles (SVP) are also secreted. These SVP are formed as a by-product of virion assembly and contain a lipid membrane and prM-E complexes but lack a capsid[19].

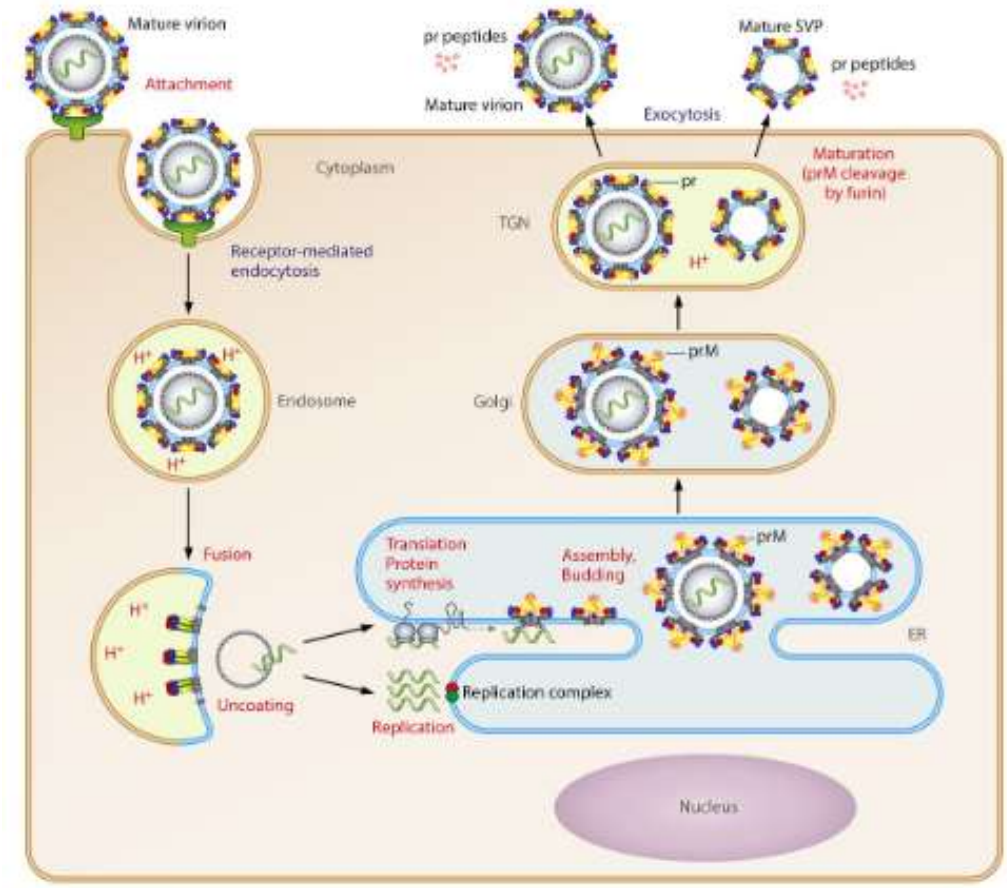


Figure 3. The flavivirus life cycle.

Virus enter cell by receptor-mediated endocytosis. The acidic pH in the endosome triggers the fusion of endosome and virus membrane resulting in the viral genome entering the cytoplasm. Following replication of viral RNA and proteins, assembled immature viral particles in the ER travel through the TGN and undergo to viral maturation process by cleavage of the pr fragment by furin before budding out of the cell. Figure reprinted from reference[19].

Epidemiology

JEV is an arbovirus (arthropod borne virus) and it is transmitted by mosquitoes of genus *Culex.* In particular, *Culex tritaeniorhynchus* and *Culex vishnui* are considered the main vectors in Asian countries, because of their propensity to breed in rice paddies and other dirty stagnant water[66]. The virus is maintained in the environment through an enzootic cycle that involves waterbirds, such as herons and egrets. Pigs play a crucial role in transmission to humans by acting as amplifying hosts. Despite the fact that pigs do not develop symptoms following JEV infection (although pregnant sows can experience abortion), they present prolonged high viraemia so are infectious to

feeding mosquitoes. Pigs are kept close enough to human dwellings to facilitate the transmission of JEV to humans. In countries where the density of pigs is low, domestic birds may contribute to JEV transmission to humans[67]. Humans are considered dead-end hosts as they develop a transient and low viraemia and therefore they are not able to transmit the virus to mosquitoes[66].

JEV is considered to be one of the main causes of encephalitis in Asia, with 100,308 JE cases and 25,125 death estimated in 2015[68].

JE is mostly a disease of children with the highest incidence among age 3-6 years. Serological surveys in endemic areas showed a high prevalence of neutralising antibody (NAb) in adults suggesting immunity in this age group presumably resulting from previous infection. However when the virus is introduced into new regions, adults are also affected [3].

Two major patterns of JE are recognised: epidemic and endemic. Epidemics of JE occur mainly during the summer in the North Asian countries (23–43° N), whereas in South Asian countries (1–13° N) JE is considered endemic[6]. Furthermore, climatic conditions seem to correlate with JE outbreaks with high temperature and low rainfall being favourable to the transmission of JEV to humans[69].

Tracking the spread of JEV over time is challenging. Since the first JE epidemics in Japan in 1871, the virus has spread to all countries of South and South-East Asia. In 1995, a serological survey identified the presence of JEV NAb also in the Northern region of Australia[70].

Different measures like mass vaccination, use of pesticides, improvement of pig rearing practice, implementation of air conditioning inside houses and a reduction of mosquito breeding places have been undertaken by different countries to prevent transmission of JEV to people. These strategies have been successful; for example although the virus still circulates in Japan, South Korea and Taiwan, these countries have seen a large reduction, and virtual elimination, of the number of JE cases[71]. Interestingly, following widespread introduction of JE vaccination, Taiwan, Japan and South Korea are facing a shift in age population of JE patients with majority of JE cases occurring in adults[15 16].

Based on the full length genome, JEV are classified into 5 genotypes (GI-V). GI and III are the most prevalent as they are found in all countries where JEV is endemic. More recently, GI has replaced GIII as the dominant genotype[74]. GII, initially isolated in Malaysia, has also been identified in Indonesia, Papa New Guinea, Australia, South Korea, Thailand and Japan. GIV has only been isolated from mosquitoes in Indonesia[75]. The first GV strain was isolated from a patient in Malaya. Subsequently, GV isolates were obtained from mosquitoes in China and South Korea[75].

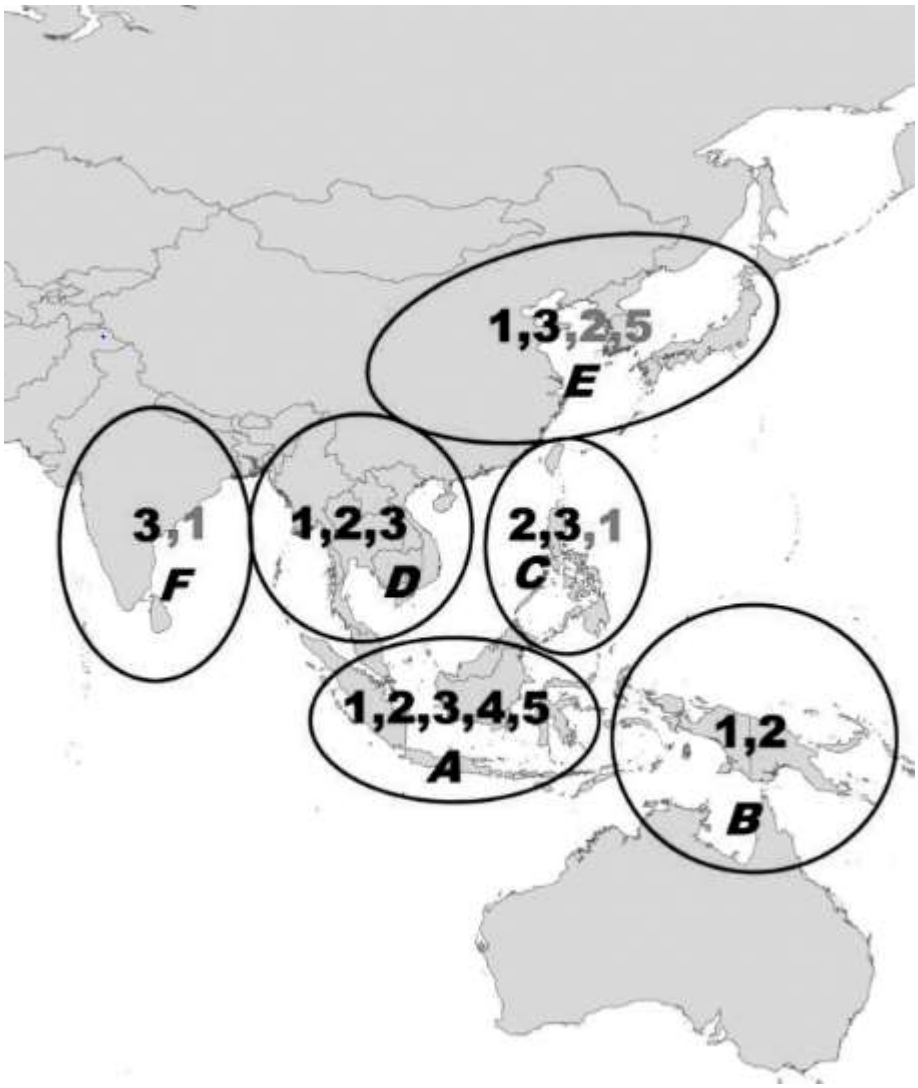


Figure 4. Geographic distribution of the 5 JEV genotypes.

A, Indonesia (excluding New Guinea) and Malaysia; B, Australia and New Guinea; C, Taiwan (China) and the Philippines; D, Thailand, Cambodia, Vietnam, Laos, and Myanmar; E, China, Japan, and South Korea; and F, India, Sri Lanka, and Nepal. The numbers in black are the genotypes prevalent before 2000, while grey numbers represent the genotypes which emerged after 2000. Figure reprinted from reference[75].

The first evidence of JEV in India goes back to the 1950s in Vellore, in the southern state of Tamil Nadu[76]. Although JE was later described in different states, the first major JEV outbreak occurred in 1973 in West Bengal (North state of India). In more recent years, although there are 24 endemic states for JEV, Uttar Pradesh accounts for more than 75% of JE cases[77]. Following the starting of the JE vaccination campaign in 2006 with the live attenuated JE vaccine (LAJV) SA14-14-2, some studies reported increasing number of JE cases[78]. Particularly significant was also the observation of a shift of the JE cases from children to adults with a ratio of 1:2.2/3.8[78–80]. However the possibility that the increase of JE cases is a reflection of an improved surveillance system and diagnosis of JE in India cannot be fully excluded. Finally, the reason of the shift of JE cases requires further investigation. In fact, although previous work has shown reduced immunogenicity of the SA14-14-2 in Indian adults (described below)[81] a lower vaccination coverage among adults in comparison to children could also account for this shift of JE cases[82].

Vaccines

Prevention of JE can be achieved by vector control or vaccination. As mosquito control is not practical to achieve in many areas, efforts have been directly at developing JE vaccines. Different vaccine technologies have been applied to develop safe and immunogenic JE vaccines.

Mouse brain derived vaccines

The first vaccine against JEV was developed by BIKEN (Japan); it was licenced in Japan in 1954 and marketed as JE-VAX. This vaccine was also manufactured by the Korean Green Cross. JE-VAX was produced by infecting intra-cerebrally 3-4 weeks old mice with JEV, the supernatant of mouse brain homogenate containing the virus was then treated with formalin and the inactivated virus purified by ultracentrifugation. Two different GIII strains were used: Nakayama and Beijing-1. The latter strain was originally isolated from the brain of a patient in 1949. The vaccine showed 91% efficacy as a monovalent (Nakayama strain only) or bivalent (containing Nakayama and Beijing strains) preparation[83]. In this clinical trial, which was conducted in Thailand, a flavivirus endemic country, the vaccine was administrated as 2 dose schedule. On the contrary, three doses are recommended for travellers[84].

For many decades JE-VAX was the only vaccine available and it has been used in different countries, including China, South Korea, Taiwan and Thailand. Although this vaccine has brought down the number of JE cases in many countries, it has several drawbacks leading to the development of new and better vaccines. Major disadvantages include: the requirement for multiples doses, poor long-term immunogenicity requiring booster doses, prohibitive costs for many countries and side effects. Local side effects were very common and systemic side effects occurred in approximately 10% of vaccinated people. Development of acute disseminated encephalomyelitis (ADEM) was the most important risk linked with this vaccine, although investigations looking for mouse myelin basic protein in the vaccine preparation were negative. In 2005 a child in Japan developed ADEM following JE vaccination, in reaction the Japanese Government suspended the use of this vaccine. In the same year, the production was interrupted and all the remaining stock expired in 2011. However, there is no firm evidence that supported a causal link between JE-VAX and ADEM[85].

Cell culture derived inactivated vaccines

This class of vaccines includes 5 different vaccines, which are summarised in table 1.

Table 1. Cell culture derived inactivated vaccines

Vaccine	**Cell type**	**Strain**	**Manufacturer**	**Year licenced**
JEBIK	Vero	Beijing-1	BIKEN	2009
ENCEVAC	Vero	Beijing-1	Kaketsuken	2011
JEVAC	Vero	P3	Liaoning Chengda Biotechnology Co	2008
JENVAC	Vero	Kolar-821564XY	Bharat Biotech	2013
IXIARO	Vero	SA-14-14-2	Intercell	2009

Two cell culture derived vaccines were developed using the Beijing-1 strain grown on Vero cells and then inactivated. The first one was marketed in 2009 by BIKEN (JEBIK) and the other one in 2011 by Kaketsuken (ENCEVAC). JEBIK and ENCEVAC were both shown to be safe and immunogenic[86,87].

JEVAC is an inactivated Vero cell derived vaccine using the Beijing P-3 strain. In a phase III clinical study conducted in children in Thailand, the vaccine, given as 3 dose schedule, was safe and highly immunogenic[88].

JENVAC is also a Vero cell derived which makes use of the Indian strain Kolar-821564XY. A phase 2/3 clinical trial conducted in India showed a seroconversion rate (SCR) of 96.9% 8 weeks after vaccination with a 2 dose schedule [81].

The most used inactivated vaccine is a Vero cell derived vaccine marketed as Ixiario which makes use of the attenuated strain SA14-14-2. It is currently approved in Europe, the USA, Canada, Australia, New Zealand, Hong Kong, Singapore and Israel. The vaccine has been licenced as a 2 dose regimen and it has been proved to be safe in children and adults. The SCR in elderly was only 65% at day 70 in comparison to >99% observed at day 56 in children[89–92].

Live attenuated vaccines

There are two live attenuated vaccines: the live attenuated SA14-14-2 vaccine and the chimeric JE vaccine.

The live attenuated SA14-14-2 vaccine was derived from serial passages of the parent strain SA14 in primary hamster kidney cells, non-neural tissues of mice and Syrian hamsters and suckling mice. Sequencing of the viral genome of the vaccine and the parent strain demonstrated about 57-66 nucleotide changes which resulted in 24-31 AA changes[93]. Nucleotide changes were found in all the viral genes except prM. Although the molecular mechanisms of attenuation are not fully understood, particularly important for neurovirulence seems to be the AA substitution G244E[94]. Moreover, the nucleic acid mutation G66A in NS2a was demonstrated to be critical for reducing neurovirulence and neuroinvasiveness of the parent strain by ablating the production of NS1′ [95]. The LAJV SA14-14-2 was initially licenced in China in 1988. A two dose vaccine schedule was safe and showed increased immunogenicity over one dose, the SCR being around 80-99%[96–98]. The efficacy of LAJV has been

estimated in case control studies, showing that a single dose of the vaccine reduced JE cases with an efficacy between 96.2% and 99.3%[99,100]. Another study however demonstrated that the efficacy of a single dose of the vaccine was 80% whereas efficacy of a booster dose given at 1 year after vaccination was 97.5%[101].

Some reports from India in adults described the vaccine as being poorly immunogenic. Singh *et al.* observed that seroconversion and seroprotection were 57.69% and 77.56%, respectively, on day 28 and 39.74% and 60.26%, respectively, on day 56[81]. Another report from India detected a SCR of 73.9% at 28 days after vaccination and 43.1% after 1 year[102]. Finally, a retrospective case control study conducted in India described a vaccine efficacy of 43.8% based on vaccination card or record[103].

The chimeric JEV vaccine (IMOJEV) is based on the yellow fever virus (YFV) vaccine backbone with the prM and E proteins of the SA14-14-2. The vaccine was found to be safe in humans[104]. Initial work investigated the use of the vaccine in YFV immune or naïve volunteers[105]. Although seroconversion was 100% in both groups the geometric mean titre (GMT) of NAb was higher in the YFV immune individuals, who also had higher viraemia[106]. In a phase III clinical trial conducted in adults, SCR measured with NAb ≥1:10 was 99.1% in comparison to JEVAX, which was 95.1%[104]. Several phase III clinical studies analysed the safety and immunogenicity of IMOJEV in children in JEV endemic countries[107–109]. Furthermore, long term studies demonstrated that a booster dose given up to 2 years following primary vaccination induced a SCR of 98.2% after 5 years from the booster vaccination[107,108].

The Chimeri-Vax technology has also been used to develop other flavivirus vaccines such as the dengue, West Nile and more recently a Zika vaccine.

Immune responses in viral infection

Following a viral infection, an innate and adaptive immune response get activated in order to control the invader.

The innate immune response is characterised by a rapid, non-specific, early response to a viral infection which helps limit the infection while a more specific adaptive immune response take develops. The innate immune system is able to recognise viruses as non-self through “sensors” called pattern recognition receptors (PRR). These PRR recognise pathogen associated molecular patterns (PAMPs). Some of these PAMPs are part of the infectious virus particles whereas others are products of viral

replication. PRR involved in viral infections include the toll like receptors (TLR, ten have been identified in humans) and the (RIG-I)-like receptors (RLRs). Several TLR are involved in the response to viral infections. TLR 3 recognises ds RNA, TLR 4 interacts with viral glycoproteins such as DENV NS1 triggering a pro-inflammatory immune response, TLR 7 and 8 which recognise long ss RNA and TLR 9 which responds to unmethylated CpG viral deoxyribonucleic acid (DNA)[110]. Among the RLRs, RIG-I protein detects uncapped 5′ triphosphated ds RNA whereas melanoma differentiation-associated protein 5 (MDA-5) can detect long ds RNA[110]. More recently, other PRR have been identified such as DNA-dependent activator of interferon-regulatory factors which recognises cytosolic viral DNA[111], leucine-rich repeat flightless-interacting protein 1 which binds DNA and RNA[112] and absent in melanoma 2 which promotes inflammasome activation following recognition of viral DNA[113]. Activation of PRR culminates in the production of inflammatory cytokines and antiviral type I interferons which help in controlling the infection at an early stage[110]. Another important component of the innate immune response in viral infections is the complement system which is a group of proteins that act sequentially promoting: a) lysis of enveloped viruses by the formation of pores, b) phagocytosis of viral particles through binding to viral protein and promoting opsonisation c) stimulation of the inflammatory response to foreign antigens.

The adaptive immune response consists of antibodies and T cells which are tailored for the invading pathogen. Antibodies are produced by plasma cells which differentiate from activated B cells. A total of 5 different classes of antibodies have been identified: immunoglobulin M (IgM), IgD, IgG, IgA and IgE. IgM and IgD are initially expressed on the surface of immature B cells. Following B cell activation by recognition of an antigen through the surface immunoglobulins that function as the B cell antigen receptor, B cells differentiate into plasma cells and start secreting IgM (which are secreted as pentamer), the first antibodies produced. Subsequently, interaction between activated B and T cells stimulate the processes of affinity maturation and class switching which enables the production of antibodies of alternative classes and with higher affinity. IgA (secreted as monomer or dimer) are mainly found in mucosal tissues, IgE (secreted as monomers) are mainly involved in allergic diseases and defence against parasites. Both IgA and IgE cannot activate the complement. Finally, IgG (secreted as monomer) are mainly found in the bloodstream

and can pass the placenta providing protection to the fetus as well[114]. Antibodies provide protection against viral infection through 3 different mechanisms: 1) they can directly neutralise the virus, 2) they promote viral clearance through antibody dependent cell mediated cytotoxicity (ADCC) or opsonisation of viral particles bound to antibodies, 3) antibodies bound to viral particles can promote complement activation inducing inflammation and lysis of viral particles[114].

T cells recognise antigens through their T cell receptor (TCR). However, T cells can only recognise peptide epitopes presented to them by major histocompatibility complex (MHC) proteins. T cells fall into functional subsets defined by expression of the surface markers CD4 and CD8. CD4 T cells recognise peptides (approximately 12-25 AA) presented to them by MHC II molecules whereas CD8 T cells recognise peptides (8-12 AA) presented to them by MHC I. CD4 and CD8 T cells have different roles. The primary role of CD8 T cells is to kill virus infected cells recognised through MHC I loaded with viral peptides. CD4 T cells are further subdivided into different subsets which are derived from different stimuli provided by antigen presenting cells (APC). These subsets include T helper 1 (Th1) cells, which promote the clearance of intracellular pathogens by enhancing the killing efficacy of macrophages and proliferation of CD8 T cells. Th2 cells are important in fighting extracellular parasites such as helminths and allergic diseases by promoting class switching to IgE and upregulation of high affinity receptors for IgE on basophiles and mast cells. Th17 cells help controlling bacterial and fungal infection by recruitment and activation of neutrophils. Other subsets are follicular helper T cells which interact with B cells promoting differentiation in antibodies secreting cells and inducing the formation of memory B cells and regulatory T (Treg) cells which suppress immune responses and inflammation. MHC I and II are expressed on different cell types and bind to their target peptide in different intracellular compartments. MHC I molecules are expressed on all nucleated cells and bind peptides derived from degradation by the proteasome in the ER. MHC II molecules are expressed on APC and bind peptides in the endo-lysosome where internalized proteins are degraded. However, through a process known as cross-presentation, peptides derived from intracellular proteins are able to be loaded on MHC II molecules and MHC I molecules can bind peptides derived from extracellular proteins[114].

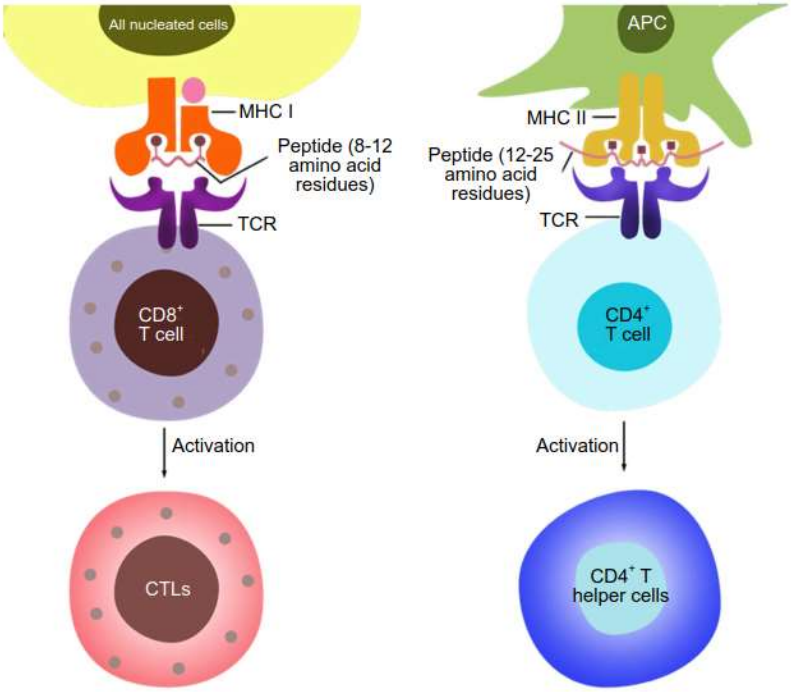


Figure 5. Differences of TCR between $CD8^+$ and $CD4^+$ T cells.

$CD8^+$ T cells recognize peptides (8-12 AA derived from degradation of an endogenous protein) loaded on MHC I molecules. $CD4^+$ T cells recognize peptides (12-25 AA derived from an internalized and degraded protein by APC) loaded on MHC II molecules. Figure reprinted from reference[115]*. CTLs: cytotoxic T lymphocytes.*

Immune response to JEV and pathogenesis

The pathogenesis of JEV is not clearly understood and much knowledge is based on studies in mice. Following JEV infection in the skin, JEV replicates in local lymph nodes, probably, by analogy with dengue virus[116], in dendritic cells[117]. The initial innate immune response seems to be critical in determining the subsequent course of infection. TLR 3 knockout (KO) mice and mice lacking the type I interferons receptor[118] are more susceptible to JEV infection. On the other hand, TLR4 KO mice were more resistant to virus replication, accompanied by a rapid increase in type I interferons[119]. However, a clinical trial conducted on JE patients showed no improvement in the outcome in comparison to placebo control when interferon α was administered[120]. A possible explanation for this failure is that, type I interferons are useful in controlling JEV replication only during the initial phase of infection and not when JEV has already entered the central nervous system (CNS).

Entry of JEV into the CNS is a critical step in JEV pathogenesis. Although different *in vitro* and *in vivo* animal studies tried to address how JEV enters the brain, the mechanism is still unclear. The distribution of lesions in the human brain at post-mortem supports a haematogenous route of spread to the CNS[121]. Replication of JEV in human brain endothelial cells (HBEC) was observed in an *in vitro* model of the blood brain barrier (BBB) with an increase in pro-inflammatory cytokines and apoptosis of cells[122,123]. It is interesting to note, however, that another study identified viral replication in the brain even before BBB disruption[124]. Finally, another animal study indicated that JEV passes through the BBB by endocytosis and transcytosis without active replication in the endothelial cerebral vessels[125]. Therefore, BBB breakdown may be the consequence, rather than the cause, of CNS invasion. Although the main target in the CNS is neurons[126,127] a pro-inflammatory response is also involved in damaging the CNS[121,128–130].

The humoral immune response to JEV has been well characterized in animal models. Several studies have shown that NAb are enough to protect from viral challenge. Konishi *et al.* demonstrated that passive transfer of serum from immunized mice was able to confer protection to high dose virus challenge[131]. Similarly, weanling mice from immunised mothers, which still have maternal antibodies circulating, were protected from challenge with JEV[132]. Sufficiently neutralising JEV monoclonal antibodies (mAbs) can also protect mice from JEV, ruling out any other effect of serum[133,134]. Dilutions of sera with NAb indicated that a minimum NAb titre of plaque reduction neutralisation test 50 ($PRNT_{50}$) of 1:10 is protective[135]. This has lead to a $PRNT_{50}$ of 1:10 being considered a surrogate marker for protection. As a placebo controlled trial to study the efficacy of new vaccines would be unethical, the use of this surrogate marker of protection is critical to avoid clinical trials with unpractically large sample size[136]. Additionally, the NAb response to JEV following immunisation with IMOJEV was found to neutralise several different genotypes of JEV[137]. Similarly, in humans, among JE patients, higher levels of IgG and IgM were found in cerebrospinal fluid as well as plasma of survivors vs non survivors highlighting the importance of the antibody response in JEV infection[138–140].

The main target of the NAb is the E protein. Several neutralising mAbs against EDIII have been isolated from mice[141] . In human, EDIII, although able to elicit NAb, seems

less immunodominant with majority of responses identified against EDI and EDII which showed a lower level of neutralisation[142,143].

The T cell response to JEV has been less characterized. In animal models, T cells have been implicated in protection as well as pathology. JEV was shown to impair the T cell priming by dendritic cells by modulating the adaptor molecule myeloid differentiation primary response 88 and antigen cross-presentation through MHC I and II[144–146]. Larena *et al.* demonstrated in a mouse model that both passive transfer of CD4 and CD8 T cells individually did not contribute to protection against JEV, though improved survival was observed when both T cell subsets were transferred. Interestingly, although CD8 T cells alone did not improve survival of challenged mice, higher CNS viral load was observed in CD8 depleted mice[147]. On the contrary, Jain *et al.* demonstrated a critical role for CD8 T cells in clearing virus infected cells through their lytic function and stopping the virus to enter CNS[148]. Similarly, intra cerebral adoptive transfer of T cells with cytolytic activity against JEV was shown to rescue adult mice from lethal intra cerebral JEV challenge[149]. Furthermore, a chimeric DENV vaccine (prM and E protein) with a backbone based on SA14-14-2, was able to confer protection to JEV challenge to mice. As a low NAb titre specific to JEV was detected, protection was likely to be due to T cells[150]. In other studies, CD4 T cells contribute to protection by promoting antibody production and isotype switching[151–153].

In humans, CD4 and CD8 T cell epitopes have been described. NS3 was initially described as the most immunodominant protein. Kumar *et al.* observed that the frequency as well as the magnitude of T cell responses to an NS3 epitope (AA 193–324) were lower in JE patients in comparison to healthy JEV exposed individuals[154]. Additionally, lower interferon-γ (IFNγ) production was associated with poor outcome[155,156]. More recently, a CD8 response was shown to be associated with asymptomatic JEV infection. These responses were multifunctional and preferentially recognised non-structural proteins. On the other hand, CD4 T cells targeting mainly the structural proteins dominated the response in JE patients. Furthermore, a multifunctional CD4 T cell response in convalescent JE patients was associated with more complete recovery from infection. Finally, T cell responses towards the more conserved flavivirus epitopes were observed in healthy JE exposed individuals in comparison to JE patients who were targeting less cross-reactive epitopes[157]. These data suggest an important role of T cells in controlling the virus.

The T cell response to the LAJV has not been fully studied. Recently, work published by our group described the T cell response in adult healthy volunteers vaccinated with the LAJV in India. T cell responses specific to JEV were described together with cross-reactive responses. Both CD4 and CD8 T cell responses were observed against all JEV proteins[158].

Dengue virus

Dengue virus (DENV) is another member of *Flavivirus* that is also transmitted by mosquitoes (genus *Aedes*). There are 4 different DENV serotypes that share approximately 70% of their protein sequence and cause a disease with similar clinical manifestations to each other. DENV is present in all tropical and subtropical countries, including India. All four DENV serotypes are now circulating in Asia, Africa and America[159]. Approximately 400 million infections have been estimated to occur every year with roughly 1 in 4 people infected being symptomatic[160]. Although inapparent DENV infection is associated with low viraemia, asymptomatic infections contribute to transmission of DENV as they can still transmit the virus to mosquitoes[161]. Symptoms of dengue include severe headache, retro orbital pain, muscle and joint pains, nausea, vomiting, swollen glands or rash. According to the WHO guidelines of 2009 the disease is classified as dengue with or without warning signs and severe dengue[162]. Severe abdominal pain, persistent vomiting, rapid breathing, bleeding gums, fatigue, restlessness and vomiting blood are the warning signs that herald the development of severe dengue. Established severe dengue is characterised by plasma leakage, extra-vascular fluid accumulation, respiratory distress, severe bleeding or organ impairment and is a life-threatening condition[162]. The critical stage is during or after the defervescence phase (figure 6) when the patient can rapidly progress towards a shock syndrome[163]. Fortunately, only a small fraction of dengue cases progress towards severe dengue. Many sero-epidemiological studies, including some of particular significance from Cuba and Thailand, demonstrated the major risk for severe dengue is a secondary infection, with an accompanying heterologous antibody response[164,165]. In addition, age plays an important role in susceptibility with infants of 4–9 months of age and children 5–9 years old being at higher risk of developing the severe dengue[166]. Extensive research has been done to explain the pathogenesis of dengue highlighting a very complex interplay of viral and host factors that promote severe clinical manifestations[167]. The exact mechanism of severe dengue is still not

fully clear though three main hypotheses have been formulated: ADE, original antigenic sin (OAS) and antibodies against NS1.

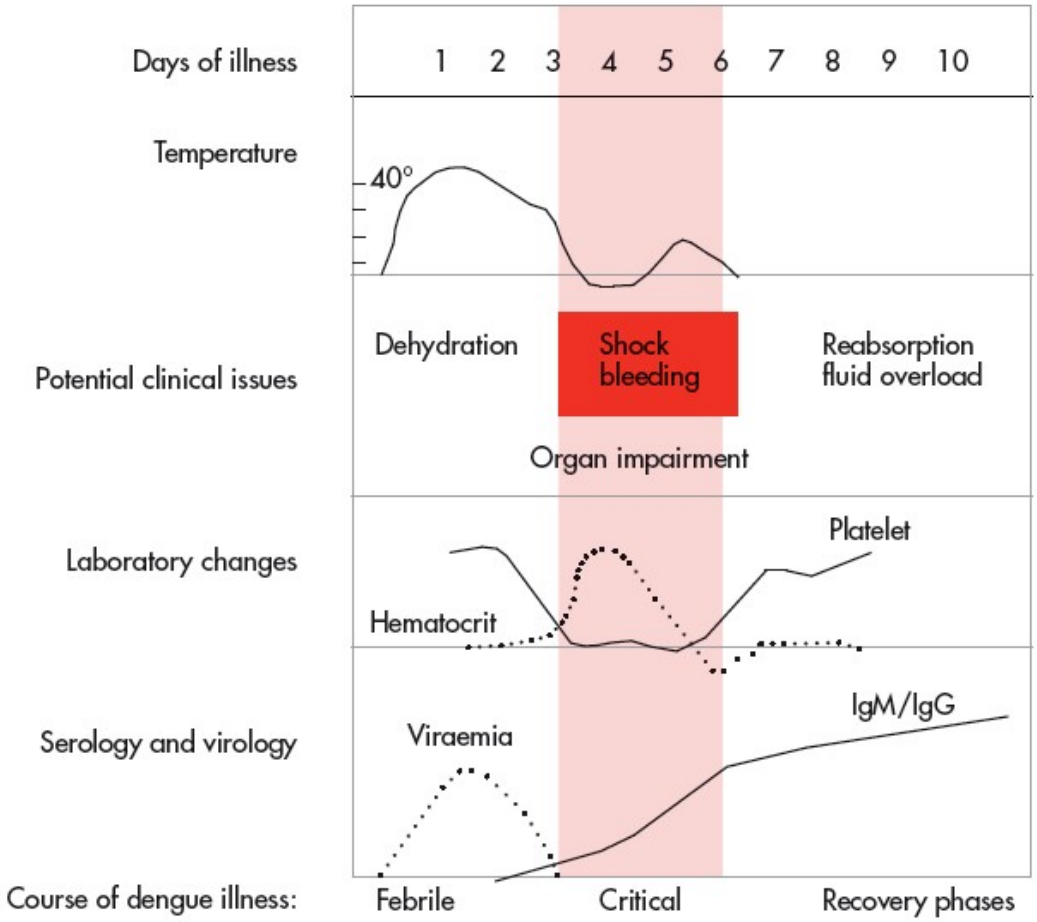


Figure 6. The course of dengue illness.

The critical phase of the disease which is life-threatening is when fever and viraemia are dropping. Shock, bleeding and organ impairment are accompanied by reduction in platelet count and increase in haematocrit. Figure reprinted from reference[162].

ADE

Following a primary infection, antibodies are raised against the infecting serotype. The targets of these antibodies include prM, E and NS1 and include both serotype specific and cross-reactive antibodies[168,169]. The primary response is thought to confer lifelong protection against the homologous serotype, though rare re-infections with the same serotype are described[170]. However, primary infection is also able to provide protection against other serotypes for the first few months[171]. These data indicate that a cross-reactive response which is also cross-protective is possible. On the contrary to what observed during primary infection, analysis of the antibody response in

secondary heterologous infection reveals that the response is dominated by cross-reactive antibodies[143,172,173]. The hypothesis of ADE suggests that some cross-reactive antibodies are poorly neutralising and promote infection of Fc receptor bearing cells by DENV[174,175] (figure 7). The *in vitro* data indicated that low concentrations of all antibodies are able to promote ADE[143]. More recently, higher incidence of severe dengue following a heterologous infection was shown in patients with an anti-DENV antibody titre ranging from 1:21 to 1:80[176]. At the molecular level, this can be possibly explained by the work of Chan *et al.*[177]. At high concentration of antibodies, large immune complexes may form. These immune complexes will interact with FcRIIb which is expressed at lower levels than FcRI and IIa and inhibits phagocytosis. ADE is thought to contribute to increased severity not only by increasing infectivity of DENV on Fc receptor bearing cells but also by promoting suppression of the type I interferon response and by increasing the expression of the anti-inflammatory cytokine IL-10 in monocytic cell lines[178,179]. However, increased expression of IL-10 and suppression of type I interferons under ADE condition was not observed with primary human monocytes[180].

Study of anti-DENV mAbs helped in understanding the humoral immune response to DENV and the mechanism of ADE. Antibodies which target prM were found to be highly cross-reactive; these antibodies were poorly neutralising even at high concentrations but could promote ADE[181]. Considering the inefficiency of the process of cleavage of the pr fragment and the production of mature, partially mature and immature viral particles during viral replication, this study also indicated that even fully immature viruses (which are not normally infectious) could be rendered infectious through ADE by anti-prM antibodies. Another important class of cross-reactive mAbs described are those able to bind the FL of E protein[182]. Although the FL plays a crucial role during the entry step of infection and it is highly conserved among flaviviruses, antibodies that bind it generally have poor neutralising activity. On the contrary, they can enhance DENV infection[183]. Moreover, as observed for anti-prM antibodies, anti-FL antibodies also had the ability to bind and promote ADE of fully immature viral particles. Serotype specific neutralising mAbs have been described which bind to more complex quaternary epitopes[184]. For example, the human mAb 5J7 could only strongly neutralise DENV 3 binding to 3 different domains of E and each one of them was located on 3 different E proteins[185]. Another similar

mAb, 2D22, specific to DENV2, was described to bind a quaternary epitope consisting of an E dimer and possibly blocking the dimer from rearranging into a trimer[186]. Another two mAbs were described to potently neutralise DENV 1: HM14c10 and 1F4. Both these mAbs were able to bind whole virion only[187, 188].

More recently, another class of broadly cross-reactive NAb recognising a novel E dimer epitope of the 4 DENV serotypes was described. These antibodies bind the interface among the two E proteins that make the dimer, a region that includes the FL[183, 189]. These broadly cross-reactive NAbs were cloned from plasmablasts isolated from hospitalised viraemic children during secondary DENV infection.

Although ADE is thought to contribute to the severity of dengue by increasing viraemia, the most important life threatening events, plasma leakage and shock, mainly occur when the patient's fever has broken and the viraemia is dropping or there is no circulating virus (figure 6)[162]. This clearly indicates that ADE alone does not fully explain the pathogenesis of dengue.

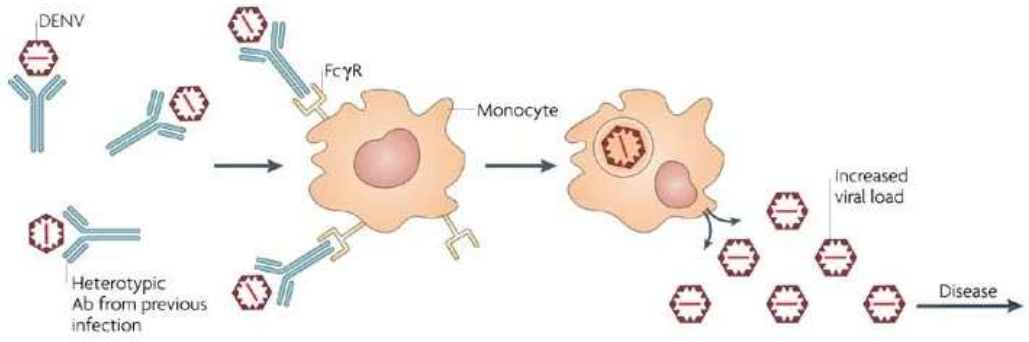


Figure 7. ADE in DENV infection

Pre-existing heterotypic antibodies raised during the primary DENV infection may bind but poorly neutralise the virus during a secondary heterologous DENV infection. These virus-antibody complexes are internalized by Fc receptors bearing cells promoting their infection. Figure reprinted from reference[190].

Original antigenic sin

The phenomenon of OAS was initially described by Dr Thomas Francis Jr. in "On the doctrine of original antigenic sin" in 1960[191]. His hypothesis was based on serological data from three different age groups of volunteers who were vaccinated with different strains of monovalent influenza vaccine. He observed that before vaccination, in each

age group the highest antibody titre against the influenza was against the strain that had been circulating during the childhood of that group. The response observed following vaccination demonstrated that despite developing an immune response towards the strain used for vaccination, the highest antibody response was newly detected against the virus strain that was circulating during the childhood for each age group[192]. In addition, animal models of influenza virus demonstrated that OAS can reduce the efficiency of vaccination measured by reduced antibody level and increase in viral titre after infection[193]. Interestingly, the degree of OAS induced depended on the type of vaccine used with inactivated vaccine inducing the phenomenon only minimally in comparison to a DNA vaccine (medium level of OAS) and live attenuated vaccine (high level of OAS). Analysis of antibody responses that exhibit OAS were however described to be important and partially promote protection[194]. OAS was also observed in different animal models and in humans for other infectious diseases such as chlamydia trachomatis and dengue[195,196]. OAS was later shown to exist in T cell responses as well, and impaired the cytotoxic T cell response and viral clearance in a mouse model of lymphocytic choriomeningitis virus[197].

The mechanism underlying OAS has not been identified yet although some hypotheses have been formulated. One hypothesis relies on competition among naïve and memory B cells for epitopes. As memory B cells have a lower activation threshold and higher cell frequency outcompete naïve B cells[193]. Another hypothesis proposed by Ndifon suggested a role for Treg in the development of OAS. Treg raised by the original antigen get activated during the exposure to the second antigen limiting the amount of antigen loaded on APC and therefore reducing the activation of naïve B cells[198]. Both hypothesis are supported by observations that repeated stimulation with the secondary antigen or the use of adjuvants to stimulate APC can overcome OAS[199]. Neutralisation of interleukin (IL)-10 (an anti-inflammatory cytokine) can also block OAS although in the study Treg were not the source of IL-10[200]. Although these models require experimental validation other questions remain unanswered such as if there is a relation between T cells and antibodies with OAS and the consequences of the phenomenon of OAS on severity of disease or impairment of vaccine efficacy (and not simply antibody levels) in humans.

In fact, in humans there is lack of evidence of OAS being involved in increased severity of a disease or decreasing vaccine efficiency. The only evidence may come

from unpublished data from the human papilloma virus (HPV) vaccine (Gardasil 9), which is supposed to give protection against 9 serotypes of HPV. Vaccination with Gardasil 9 of individuals previously vaccinated with an earlier version of the vaccine (Gardasil, which protected only against 4 serotypes) was less efficient in volunteers vaccinated with Gardasil 9 alone[201]. Antibody titre against the 5 HPV serotypes not in common with the previous version of the vaccine were observed in 25% in the group that received both vaccines vs 69% observed in the volunteers who received Gardasil 9 alone. However, the clinical significance of this observation is unknown.

OAS was also initially demonstrated in the antibody response to DENV infection and subsequently for T cell responses[202]. The T cell response to DENV has been extensively studied in animal models as well as in human studies. Initial work proposed involvement of T cell responses in the pathogenesis of dengue. Although T cell epitopes were found in all dengue proteins, NS3 was immunodominant[203,204]. Together with serotype specific T cell responses, cross-reactive T cells were also identified with the ability to kill cells pulsed with heterologous dengue peptides[205]. One study demonstrated a higher magnitude of T cell responses but decreased killing capacity (measured by the staining CD107a as a marker of degranulation) in dengue haemorrhagic fever (DHF) patients[203]. The observation of OAS in the T cell response to DENV[202], together with the description of altered and less vigorous responses with decreased cytotoxic activity towards heterologous dengue peptides variants[205–207], led to speculation of involvement of OAS in the pathogenesis of dengue. During a secondary heterologous DENV infection, OAS could promote the proliferation of T cells specific to the serotype of the primary infection and a skewed cytokine profile that lead to a cytokine storm with subsequent DHF and shock[208] (figure 8). However, OAS was subsequently observed as a general phenomenon occurring in a dengue endemic population and therefore could not explain severe dengue, a rare progression of the disease[209,210]. Additionally, several studies indicated that the T cell response may play an important role in protection. Hatch *et al.* described higher frequencies of dengue specific T cells in school children who developed asymptomatic dengue in comparison to those who developed clinical manifestation[211]. Furthermore, animal studies corroborated the protective role of CD8 T cells[212,213]. In humans, HLA molecules associated with higher magnitude of response rather than breadth of frequency were associated with protection[209]. An interesting study by Dung *et al.*

demonstrated that in patients with DHF the starting of haemoconcentration and thrombocytopenia occurred before the appearance of detectable CD8 T cells specific to a dengue epitope[214]. These data may indicate that the T cell response may contribute to both protection and pathology, and is not the only factor contributing to the pathogenesis of dengue.

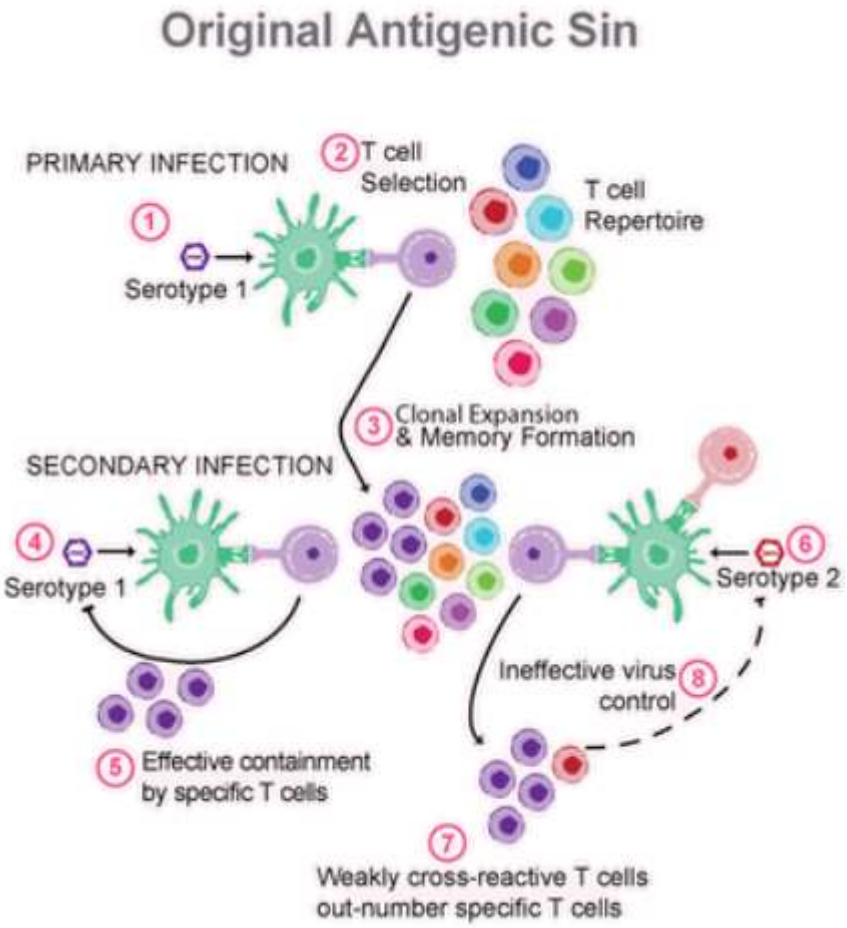


Figure 8. OAS in T cells in dengue

During a secondary heterologous DENV infection, T cells raised during a primary DENV infection can get activated due to the phenomenon of OAS. These cross-reactive T cells out-number T cells specific to the serotype of the secondary infection and may promote a suboptimal response which can lead to a cytokine storm. Figure reprinted from reference[215]

NS1

The main feature of severe dengue is the development of plasma leakage, which is due to increased vascular permeability and can lead to shock. This is a transient state, and is believed to be induced by soluble mediators such as cytokines or chemokines.

Recently, two independent groups highlighted the importance of NS1 in promoting plasma leakage in a mouse model[216,217]. Inoculation of NS1 alone was sufficient to induce vascular permeability and production of pro-inflammatory cytokines and chemokines[216,217]. NS1 was observed to work as a PAMP interacting with TLR4 and triggering a strong cytokine production[217,218]. *In vitro* studies demonstrated that NS1 increased endothelial cell permeability directly by disrupting the cell glycocalyx (a matrix made of glycoproteins and glycolipids that surrounds the cell membrane) even in the absence of cytokines[219,220]. Additionally, administration of antibodies against NS1 or immunisation with NS1 was able to protect mice from viral challenge indicating an important role of NS1 in the pathogenesis of dengue[216,221]. To a lesser extent, protection was also observed when mice were immunised with a NS1 of a different serotype of the challenge virus[216].

Another consistent characteristic of severe dengue is thrombocytopenia (reduction of platelet count). Reduction in platelets during DENV infection may be mediated through multiple mechanisms. DENV can reduce the production of platelets through infection of megakaryocytes and suppression of their precursors in the bone marrow[222–224]. Moreover, the humoral response to NS1 was also demonstrated to induce antibodies that are able to bind to self-antigens such as platelets, plasminogen and endothelial cells[225–227]. Higher titres of these autoantibodies were observed in patients with severe dengue.

Dengue vaccine

The only dengue vaccine currently approved in several endemic countries is Dengvaxia. Dengvaxia was developed by Sanofi Pasteur and is a tetravalent chimeric live attenuated dengue vaccine. The backbone of the vaccine is the yellow fever vaccine 17D which expresses the prM and E proteins of each DENV serotype[228]. A phase I and II clinical trials demonstrated the vaccine is safe and can elicit NAb against all DENV serotypes[229–233]. Data from a phase IIb and two phase III clinical trials (one conducted in Latin America and one in Asia) demonstrated differences in vaccine efficacy against the 4 serotypes with the highest efficacy observed against DENV4 and the lowest against DENV2. Analysis of the data highlighted also that the vaccine was less efficient in volunteers less than 9 years of age[234–237]. Furthermore, some evidence suggested that the vaccine can increase hospitalization in seronegative individuals of any age group[238].

Flavivirus cross-reactivity

Studying the epidemiology of flaviviruses is critical for public health as highlighted by the emergence of flaviviruses in new areas of the globe in the past 20 years. In 1999 WNV reached the USA for the first time[239]. More recently, ZIKV reached Yap Island in 2007 and from then started spreading reaching French Polynesia in 2013 and Brazil in 2015, causing a large outbreak associated with the recognition of more severe disease syndromes. Since then, improved surveillance in other countries has demonstrated global spread of ZIKV with cases detected in several African and Asian countries[240].

This spread of flaviviruses into new areas creates new challenges that must be addressed. Flavivirus cross-reactivity is well known and it has important implications from a clinical and diagnostic point of view.

Cross-reactivity among different flaviviruses has been studied previously. Although not statistically significant, Hoke *et al.* described a reduced attack rate and severity of dengue fever and DHF in volunteers receiving JEVAX vaccine[83]. Cross-protection was also observed in an animal model, when mice immunised with an inactivated or a LAJV were challenged with DENV[241]. Furthermore, live attenuated flavivirus vaccines have increased immunogenicity in people with pre-existing antibodies to a different flavivirus[242–244]. The increased immunogenicity of these vaccines may possibly due to an ADE effect that increases viraemia and stimulates a pro-inflammatory immune response. Cross-reactive ADE from different flavivirus immune sera was demonstrated in *in vitro* studies with contrasting results[245,246]. *In vivo* effects are also described; recently, Bardina *et al.* demonstrated in a STAT2 KO mouse model an enhanced effect of DENV and WNV immune sera on ZIKV challenge[247]. In humans, Anderson *et al.* described a higher frequency of symptomatic dengue in people with pre-existing NAb to JEV, but no NAb to DENV. However, no difference in the presence of NAb against JEV was observed among people who developed DHF[248].

Work focusing on flavivirus cross-reactive T cells has also been conducted. Cross-reactive T cell clones raised from DENV infected donors were demonstrated to react and kill target cells pulsed with WNV antigen[249]. Similarly, JEV specific T cell clones could proliferate following WNV antigen stimulation[250]. Finally, following the recent

outbreak of ZIKV, many studies demonstrated important T cell cross-reactivity between ZIKV and other flaviviruses[251–253]. A few studies also focused on the implications of cross-reactive T cells in disease. In a mouse model, T cells specific for DENV could reduce viraemia in several organs in a ZIKV challenge[254]. In humans, T cell responses in DENV exposed individuals were shown to alter the T cell response to ZIKV infection. This influence was observed not only in terms of kinetic of the response but also in terms of magnitude, with an earlier and more vigorous T cell response observed in DENV exposed individuals. Furthermore, following ZIKV infection, a pre-existing DENV T cell response influenced the pattern of recognition of ZIKV proteins, with structural proteins targeted in DENV naïve volunteers in comparison to DENV immune subjects who preferentially targeted the non-structural proteins[255].

Aims

Since the use of the live attenuated Japanese encephalitis (JE) vaccine (LAJV) in India a shift to relatively increased infections in adult population was observed. Some studies have demonstrated a reduced immunogenicity of the LAJV in Indian adults. India is endemic to both Japanese encephalitis virus (JEV) and dengue virus (DENV). In this project, 17 adult healthy Indian volunteers were vaccinated with a single dose of the LAJV. The project aims were:

- Determine the immunogenicity of the vaccine and related it to previous DENV exposure
- Determine if original antigenic sin occurs following vaccination in DENV seropositive participants and analyse it in relation to the immunogenicity of the LAJV
- Identify T cell responses to JEV after vaccination, analyse their cross-reactivity with DENV variants and determine if they were present before vaccination
- Relate the T cell and antibody responses

Chapter I: Materials and methods

Viruses

Japanese encephalitis virus (JEV) SA14-14-2, dengue virus (DENV) 1 16007, DENV2 16681, DENV3 16562 and DENV4 C0036/06 were obtained from Armed Forces Research Institute of Medical Sciences (AFRIMS).

Cell lines

C6/36 (*Aedes Albopictus*, larva) and LLC-MK2 (*Macaca mulatta*, monkey, rhesus, kidney) cell lines were obtained from AFRIMS.

Cell culture media

All cell culture media are described in the tables below (table 2-10):

Table 2 Constituents of growth media for LLC-MK2.

LLC-MK2 growth media	
Reagent	**Concentration**
M199	NA
Fetal calf serum (FCS) (heat inactivated)	10%
HEPES	20mM
L-glutamine	2mM
Penicillin/Streptomycin (P/S)	100U/ml and 0.1mg/ml
$NaHCO_3$ (7.5%)	0.6%

Table 3. Constituents of viral diluent.

Viral diluent	
Reagent	**Concentration**
Minimum essential medium (MEM)	NA
FCS (heat inactivated)	10%
L-glutamine	2mM
P/S	300U/ml and 0.3mg/ml
$NaHCO_3$ (7.5%)	1%
pH was adjusted to 7.4-7.8	

Table 4. Constituents of Hank's balanced salt solution (HBSS).

HBSS	
Reagent	**Concentration**
Sterile mQ water	NA
HBSS 10x	10%
P/S	100U/ml and 0.1mg/ml
$NaHCO_3$ (7.5%)	0.4%
pH was adjusted to 7.3-74	

Table 5. Constituents of first overlay.

First overlay	
Reagent	**Concentration**
Sterile dH_2O	NA
HBSS 10x	20%
FCS (heat inactivated)	10%
MEM Vitamin solution 100x	2%
MEM amino acid (AA) 50x	2%
L-glutamine	2.4mM
P/S	200U/ml and 0.2mg/ml
$NaHCO_3$ (7.5%)	4%
pH was adjusted to 8.2 by adding 2M solution of NaOH	

Table 6. Constituents of second overlay.

Second overlay	
Reagent	**Concentration**
Sterile dH_2O	NA
HBSS 10x	20%
MEM Vitamin solution 100x	2%
L-glutamine	2.4mM
MEM AA 50x	2%
pH was adjusted to 6.3 by adding 2M NaOH solution	

Table 7. Constituents of R10.

R10	
Reagent	**Concentration**
Roswell Park Memorial Institute (RPMI) 1640	NA
FCS (heat inactivated)	10%
L-glutamine	2mM
P/S	100U/ml and 0.1mg/ml

Table 8. Constituents of H10.

H10	
Reagent	**Concentration**
RPMI 1640	NA
Human serum AB (heat inactivated)	10%
L-glutamine	2mM
P/S	100U/ml and 0.1mg/ml
T Stim or rIL-2	10% or 50IU/ml
rIL-7	20ng/ml

Table 9. Constituents of growth media for C6/36.

C6/36 Growth media	
Reagent	**Concentration**
MEM	NA
FCS (heat inactivated)	10%
L-glutamine	2mM
P/S	100U/ml and 0.1mg/ml
MEM non-essential AA 100x	1%

Table 10. Constituents of virus infection media.

Virus infection media	
Reagent	**Concentration**
RPMI 1640	NA
FCS (heat inactivated)	5%
L-glutamine	2mM
P/S	100U/ml and 0.1mg/ml
$NaHCO_3$ (7.5%)	0.6%

Antibodies

All the antibodies used in this study are described in table 11.

Table 11. List and characteristics of all the monoclonal antibodies used in the study

Marker	Fluorochrome	Dilution used	Clone	Source
CD3	BV510	1:50	UCHT1	BD
CD4	Percp-Cy5.5	1:50	OKT-4	BD
CD8	V450	1:200	RPA-T8	BD
IFNγ	FITC	1:50	45-15	Miltenyi Biotec
TNFα	APC	1:50	Mab11	BD
CD107a	PECy7	1:100	H4A3	Ebioscience
CD19	APC-Cy7	1:50	SJ25C1	BD
CD20	Percp-Cy5.5	1:50	2H7	BD
CD27	FITC	1:50	L128	BD
CD38	PE-Cy7	1:50	HIT2	BD

Primers and probes

DENV primers and probes were described by Johnson *et al.*[256] similarly JEV primers and probe were designed by Dr Barbara Johnson (Centers for Disease Control and Prevention).

Table 12. List of primers and probes used in this study

Virus	Primer /probe	Nucleotide sequence	Position	Fluorochrome
JEV	Forward	GGCTCTTATCACGTTCTTCAAGTTT	239	NA
JEV	Reverse	ACTAGTAAGATGTTTCATTGCCACACTCT	344	NA
JEV	Probe	ATTAGCCCCGACCAAGGCGCTTT	269	FAM-BHQ1
DENV1	Forward	CAAAAGGAAGTCGTGCAATA	8973	NA
DENV1	Reverse	CTGAGTGAATTCTCTCTACTGAACC	9084	NA
DENV1	Probe	CATGTGGTTGGGAGCACGC	8998	FAM-BHQ1
DENV2	Forward	CAGGTTATGGCACTGTCACGAT	1605	NA
DENV2	Reverse	CCATCTGCAGCAACACCATCTC	1583	NA
DENV2	Probe	CTCTCCGAGAACAGGCCTCGACTTCAA	1008	HEC/BHQ-1
DENV3	Forward	GGACTGGACACACGCACTCA	740	NA
DENV3	Reverse	CATGTCTCTACCTTCTCGACTTGTCT	813	NA
DENV3	Probe	ACCTGGATGTCGGCTGAAGGAGCTTG	762	TR/BHQ-2
DENV4	Forward	TTGTCCTAATGATGCTGGTCG	904	NA
DENV4	Reverse	TCCACCTGAGACTCCTTCCA	992	NA
DENV4	Probe	TTCCTACTCCTACGCATCGCATTCCG	960	Cy5/BHQ-3

Virus growth

Initial virus titration showed a very low virus titre for all DENV stocks (180, 70, $2.16x10^4$ and $1.48x10^3$ plaque forming unit/ml (PFU/ml) for respectively DENV1, 2, 3 and 4) whereas the JEV SA 14-14-2 stock titre was $2.83x10^7$ PFU/ml (virus titres were determined by plaque assay described below) (figure 9). Regarding JEV, an 80% confluent T25 flask of C6/36 was infected with SA14-14-2 at a multiplicity of infection (MOI) of 0.1 in virus infection media with a total volume of 1ml and incubated for 1 hour at room temperature on a rocker. The MOI corresponds to the number of infectious virus particles added for each cell. At the end of the incubation, the volume was brought to 5ml and the flask incubated at 37°C 5% CO_2 till 70% cytopathic effect (CPE) was observed. To harvest the virus, virus infection media was collected and spun down at 4000 revolution per minute (rpm), 4°C for 5 minutes. The supernatant was collected and 1ml of heat inactivated FCS, 15% $NaHCO_3$ added to it. Finally, the virus suspension was vortexed, aliquoted and stored at -80°C. A similar protocol was initially used for the 4 DENV serotypes, however as the virus stocks were at lower titre (figure 9), only lower MOI could be achieved: 0.006 MOI for DENV2 and 4, 0.005 for DENV3 and 0.002 for DENV1. DENV titres measured by plaque assay remained low ($1.53x10^3$, $2.66x10^3$, $1.7x10^4$ and $1.95x10^3$ PFU/ml respectively for DENV1, 2, 3 and 4) (figure 10). A similar experiment was also carried out on LLC-MK2 for all DENV serotypes obtaining higher DENV titres ($9.3x10^4$, $1x10^5$, $8x10^4$ and $1.2x10^5$ PFU/ml respectively for DENV1, 2, 3 and 4) (figure 10). Finally, all viruses were passaged on C6/36 at an MOI of 0.1 before being used for plaque reduction neutralisation test (PRNT).

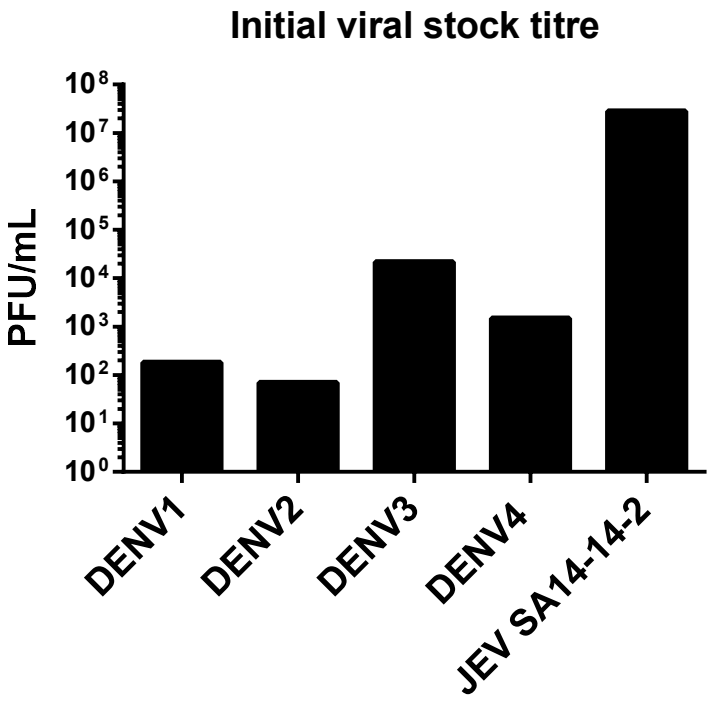


Figure 9. Viral titre of all virus stocks at the beginning of the project (grown on C6/36)

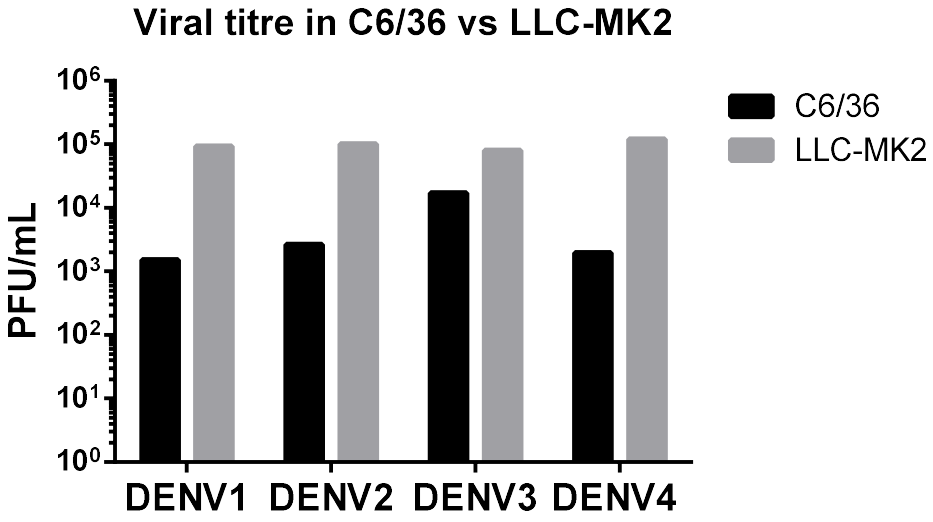


Figure 10. Viral titre of all DENV stocks grown on C6/36 vs LLC-MK2.
DENV was grown on C6/36 or LLC-MK2 at the following MOI: 0.006 MOI for DENV2 and 4, 0.005 for DENV3 and 0.002 for DENV1 and titre measured by plaque assay.

Plaque assay

Plaque assay was used to quantify infectious viral particles. The day before the assay was carried out, $1.5x10^5$ cells/well of LLC-MK2 were plated on a 12 well plate carefully shaking the plate to make an evenly distributed monolayer and incubated at 37°C, 5% CO_2 overnight. The following day, 10-fold serial dilutions of the virus were performed in viral diluent. Cells were washed once with HBSS and 200µl of viral diluent added to the cell monolayer together with 100µl of virus serial dilution (each dilution was tested in triplicate). A volume of 300µl of viral diluent was used for the negative control. Plates were then incubated at room temperature for 1 hour on a rocker. Subsequently, the viral inoculum was removed and 1ml of a 1:1 mix of first overlay and 2% low melting point (LMP) agarose added. The plate was then incubated at 37°C, 5% CO_2 for a number of days dependent on the virus tested. JEV and DENV3 were incubated for 3 days whereas DENV1 and 4 for 5 days and DENV2 for 7 days. The days of incubation for each virus were based on a protocol obtained from AFRIMS. The day before the last day of incubation 1ml of a 1:1 mix of second overlay/4% neutral red and 2% LMP agarose was added. On the last day of incubation, plaques were counted and viral titre per ml calculated by applying the following formula: average number of plaques from replicate wells x dilution factor from where the plaques were counted x 10.

Initial attempts on setting up PRNT failed to obtain a number of plaques of at least 20 in the virus control possibly due to an extra incubation step of the virus dilutions at 37°C for 1 hour required for the assay. An experiment was carried out to study the stability of the viruses at 37°C for 1h. Serial viral dilutions (10-fold) of the 5 viruses were performed and 2 aliquots generated. One aliquot was incubated at 37°C for 1 hour before being added to cell monolayer whereas the other one was directly inoculated to the cell monolayer. As shown in figure 11, while JEV was stable with a 3.6% drop, all DENV serotypes showed a reduction of viral titre: DENV2 showed the highest percentage drop (90%) followed by DENV1 (75%), DENV4 (33.3%) and DENV3 (18.2%) (figure 11). For this reason a modified version of the plaque assay was used to determine the virus titre for the stock to be used for PRNT. This modified version of the plaque assay included an incubation step of the virus dilutions at 37°C for 1 hour before adding them to the cell monolayer.

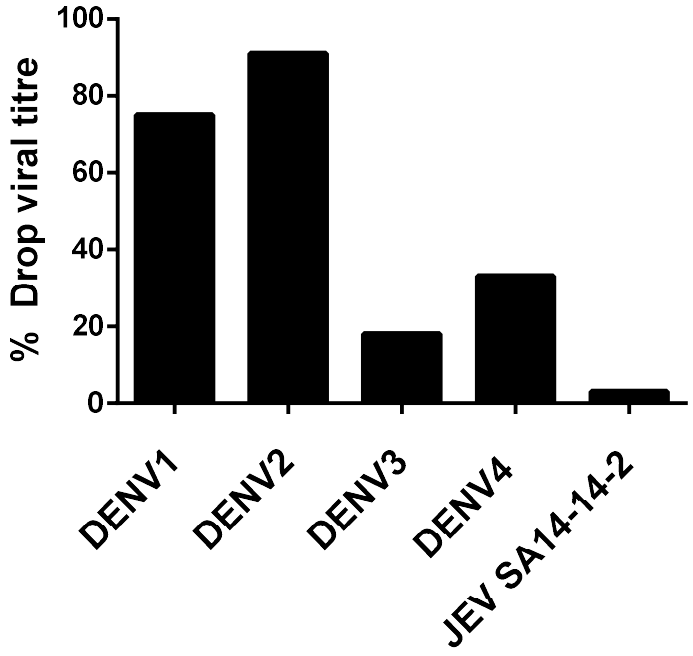


Figure 11. Stability of DENV and JEV SA14-14-2 at 37°C for 1 hour.

Percentage drop of viral titre of the 5 flaviviruses following an incubation period of 1 hour at 37°C.

PRNT

To measure the neutralising antibody titre against all the 5 viruses, PRNT were performed. The day before the assay was carried out, LLC-MK2 were plated in a 12-well plate on growth media at a cell density of $1.5x10^5$ cells/well. Starting from 1:10, five 4-fold serial dilutions of heat inactivated serum from volunteers were prepared in viral diluent. Virus stock was then diluted to 800 PFU/ml in viral diluent. This virus titre allowed to obtain a number of plaques easy to count and with minimal overlap between two or more plaques. A 1:1 solution of virus suspension and serum dilutions were prepared and incubated at 37°C for 1 hour. A viral control tube was also prepared and virus was diluted 1:1 with viral diluent. At the end of the incubation period, cells were washed with HBSS and 200µl of viral diluent added to all wells. Subsequently, 100µl of virus/serum mix was added to the cell monolayer and incubated for 1 hour at room temperature on a rocker. Finally, the inoculum was removed and a mix 1:1 of first overlay and 2% of LMP agarose added before incubating the cells at 37°C, 5% CO_2 for a number of days dependent on the virus tested. JEV and DENV3 were incubated for 3 days whereas DENV1 and 4 for 5 days and DENV2 for 7 days. The

day before the last day of incubation, a second overlay / 4% neutral red mixed 1:1 with 2% LMP agarose was added. On the last day of incubation, plaques were counted. Each serum dilution and virus control was tested in duplicate.

Polymerase Chain Reaction

A single virus was handled at time while performing virus growth to develop virus stocks and avoid cross-contamination. Additionally, after a virus stock was prepared a real time polymerase chain reaction (PCR) was performed to check for cross-contamination before using it for PRNT. Viral ribonucleic acid (RNA) was extracted by QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturer's instructions and finally eluted in 60µl of AVE buffer.

DENV RNA was amplified as four-plex using AgPath-ID™ One-Step RT-PCR reagents from Invitrogen and the reaction prepared as described in table 13:

Table 13. PCR reaction mixture for DENV RNA amplification

Reagent	Volume (µl)
2x buffer	25
Forward Primers (50µM)	0. 25 for each serotype
Reverse Primers (50µM)	0.25 for each serotype
Probes (30nM)	0.3 for each serotype
Nuclease free water	15.8
Enzyme	1
RNA	5
Total volume	50

The reverse transcription was carried out at 50°C for 10 minutes. Following an enzyme activation step performed at 95°C for 5 minutes, amplification consisted of 45 cycles of 15 seconds at 95°C (deoxyribonucleic acid (DNA) denaturation) followed by 30 seconds at 55°C. Fluorescence data were collected at 55°C.

Regarding JEV amplification, reaction was carried out in two steps: reverse transcription and complementary DNA (cDNA) amplification. Reverse transcription reaction was prepared as described in table 14 and reaction carried out for 45 minutes at 42°C.

Table 14. Reaction mixture for JEV RNA reverse transcription.

Reagent	Volume (µl)
10x reverse transcription buffer	10
25x dNTPs (50µM in 10mM Tris-HCl)	4
10x Random primers (50µM in 10mM Tris-HCl)	10
MuLv reverse transcriptase enzyme (50U/µl)	5
Nuclease free water	19
RNase inhibitor (20U/µl)	2
Extracted RNA	50

The reaction mixture for the cDNA amplification was prepared as described in table 15.

Table 15. Reaction mixture for JEV cDNA amplification.

Reagent	Volume (µl)
2x Taqman Mastermix	12.5
Forward primer (100µM)	0.125
Reverse primer (100µM)	0.125
Probe (15µM)	0.15
Nuclease free water	4.6
cDNA	7.5

The reaction consisted of: 2 minutes at 50°C followed by 10 minutes at 94°C to activate the enzyme and 40 cycles of 15 seconds at 95°C to denature the DNA and 1 minute at 60°C for annealing and amplification. The fluorescence data were acquired at 60°C.

Participants

The process of enrolment of the participants in the study was conducted earlier by Dr Lance Turtle. A total of 17 participants were enrolled and vaccinated with the live attenuated JEV vaccine SA14-14-2. Vaccine was administered either at the Indian Institute of Science or National Institute of Mental Health and Neurosciences (NIMHANS). All the volunteers were tested negative for human immunodeficiency

virus (HIV), hepatitis C virus (HCV) and hepatitis B virus (HBV). One participant withdrew from the study after a week therefore it was excluded from this study. Majority of subjects were male (76.5%) and mean age at vaccination was 26.6 years (table 16). Serum and peripheral blood mononuclear cells (PBMC) were collected before vaccination and at week 1, 2, 4, 8, 16 and 26 following vaccination by Dr Lance Turtle and cryopreserved.

Table 16. Age at vaccination of the volunteers

Volunteer ID	**Age at vaccination**
001c1k1	28
004c1k1	25
007c1k1	23
017c1k1	25
018c1k1	25
020c1k1	22
023c1k1	22
005c3k1	29
008c3k1	29
010c3k1	29
012c3k1	20
015c3k1	39
017c3k1	38
019c3k1	26
020c3k1	27
022c3k1	22
023c3k1	24

PBMC isolation and storage

PBMC were isolated and stored in liquid nitrogen by Dr Lance Turtle. Blood was collected from volunteers and diluted 1:1 with phosphate-buffered saline (PBS). Subsequently, diluted blood was slowly added to a 50ml falcon tube filled with 17ml of Lymphoprep. Subsequently, blood was centrifuged at 2200 rpm for 22 minutes with brake off. PBMCs were aspirated with a sterile Pasteur pipette from the buffy coat. A volume of 20ml of R10 was added to the cells and centrifuged at 1800 rpm for 5 minutes. Supernatant was discarded and cells re-suspended in 10ml of R10 and newly centrifuged at 1350 rpm for 3 minutes. Finally, supernatant was discarded and cells re-suspended in 10ml of R10. PBMC were stored in liquid nitrogen in the following way: cells were centrifuged at 1500 rpm for 5 min and re-suspended in ice-cold freezing media (heat inactivated FSC, 10% dimethyl sulfoxide (DMSO)) and quickly transferred at -80°C in a Mr. Frosty. On the following day, PBMC were transferred in the liquid nitrogen.

PBMC thawing protocol

PBMCs were quickly removed from the liquid nitrogen and placed at 37°C till ice crystals started melting. Subsequently, cells were transferred in pre-warmed R10 (10ml) and centrifuged at 1500 rpm for 5 minutes. Finally, supernatant was discarded and cells re-suspended in 10ml of R10.

Peptides

A peptide library covering the whole JEV SA14-14-2 proteome was purchased from Mimotopes. The peptides were 15-19 mer with 10 overlapping AA. This length was able to cover both CD4 and CD8 T cell responses. The library was organized in 11 pools and each pool consisted of 6 or 7 mini pools. Pool 1 included the peptides of capsid and pre membrane, pool 2 and 3 included peptides of envelope. Among the non-structural proteins, pool 4 included peptides of NS1 whereas peptides of NS2a and NS2b were combined in pool 5 and those of NS4a and b constituted pool 8. Finally, NS3 peptides were split into pool 6 and 7 and those of NS5 in pool 9, 10 and 11 (figure 12A). All the peptides were dissolved in DMSO and working stock of pools, mini pools and individual peptides prepared at a final concentration of 600µg/ml.

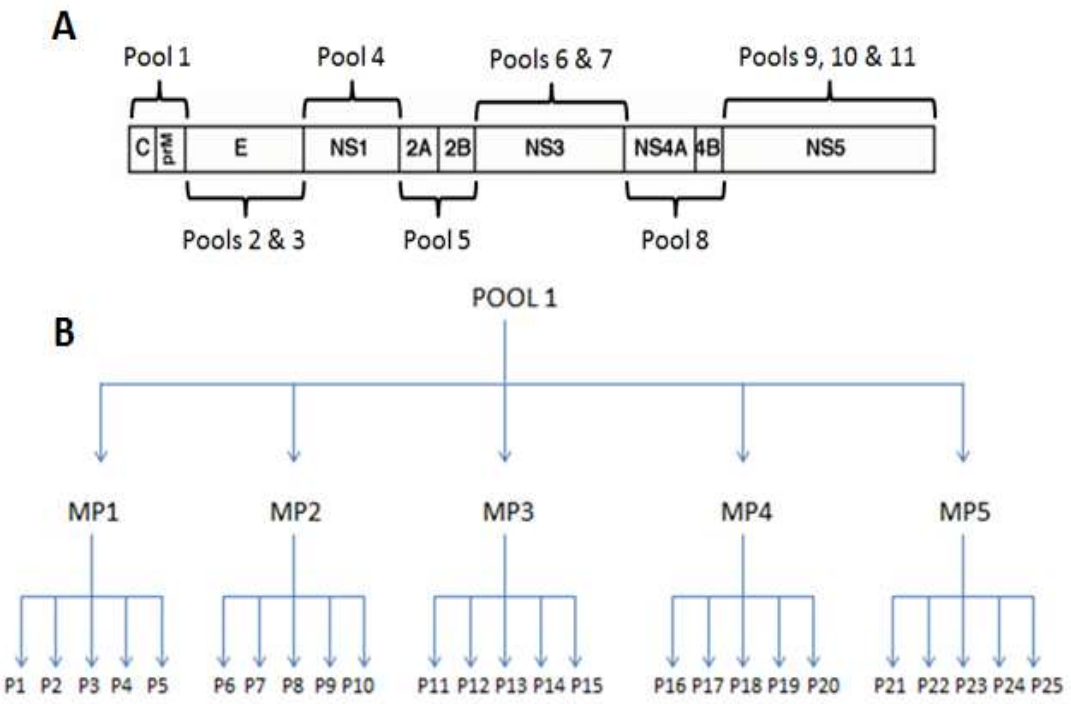


Figure 12. Schematic representation of JEV peptide library and example of mini pools organisation.

Organisation of the JEV proteins in pools (A) and example of structure of each pool in mini pools and peptides (B).

The DENV peptides were obtained from BEI resources and a full peptide library spanning the whole proteome was available for DENV2 only. Regarding DENV 1, DENV 3 and DENV 4 only the peptides of the following proteins were available: E, NS1, NS3 and NS5. The peptides were 12 to 18 mer with 11 to 13 overlapping AA and they were dissolved in DMSO at 600µg/ml. The peptides represented the following DENV strains: strain 16007 as DENV1, New Guinea C as DENV2, Philippines H87/1956 as DENV3 and Singapore 8976/1995 as DENV4.

Expansion of short term T cell lines

T cell lines were expanded from frozen PBMC. Once thawed, PBMC were rested overnight in R10 before proceeding with the stimulation. The following day, $2x10^6$ PBMC were plated in a 24 well plate in a final volume of 1ml of H10 in presence of a pool of peptides (final concentration of 3µg/ml) or mini pool (final concentration of 5µg/ml) or single peptide (final concentration of 10µg/ml). Subsequently, cells were incubated at 37°C, 5% CO_2 for 7-10 days.

Intracellular cytokines staining

On the day before the assay, cells were rested in R10 overnight. A total of $2x10^5$ cells were plated in a 96-round bottom well plate in 100µl of R10 and stimulated with mini pool or single peptide at a final concentration of 5µg/ml for 6 hour at 37°C in presence of brefeldin A (10µg/ml) (brefeldin A inhibits protein transport from the endoplasmic reticulum to the Golgi therefore blocking the secretion of cytokines). DMSO at a concentration of 0.8% was used as negative control and phorbol 12-myristate 13-acetate (PMA) (25ng/ml) and Ionomycin (500µg/ml) as positive controls. If expression of CD107a was also assessed, 1µl of anti-CD107a monoclonal antibody (details in table 11) was also added. After the incubation, cells were first stained with live dead (Near IR, BD) (diluted 1:1000 in PBS) for 20 minutes in the dark at room temperature and subsequently fixed and permeabilised with 100µl of BD cytofix/cytoperm for 20 minutes in the dark at room temperature. Cells were stained with monoclonal antibodies against CD3, CD4, CD8, IFNγ and TNFα (details in table 11) in a volume of 50µl of perm wash (1x, BD) for 30 minutes at 2-8°C and finally re-suspended in 200µl of PBS. Cells were acquired on FACS ARIA III or FACS Verse together with compensation controls and data analysed using FlowJo with the gating strategy shown in figure 13. Forward and side scatters were used to gate on lymphocytes (figure 13A) whereas forward scatter area vs height was used to exclude duplets and gate single cells only (figure 13B). Live/dead staining was performed to discriminate live from dead cells. Live/dead staining consisted of a fluorescent amino reactive molecule that is not able to penetrate the cell membrane. Therefore, dead cells which do not have an intact cell membrane allowed the amino reactive molecule to stain also internal amino groups resulting in a brighter staining. Live T cells were gated based on the T cell co-receptor multimeric protein complex CD3 (figure 13C). T cells were subsequently differentiated into CD4 and CD8 subsets (figure 13D) and responding cells were gated by analysing the secretion of IFNγ and/or TNFα (figure 13E). Finally, the ability of the T cells to kill their target cells was analysed by using the degranulation marker CD107a (figure 13F). CD107a is the lysosomal-associated membrane glycoprotein 1 (LAMP-1) which is present in the pre-formed lytic granules, containing perforin and granzymes, which are secreted during the killing process by T cells. This process exposes CD107a to the cell membrane and allows the binding of a labelled monoclonal antibody specific to CD107a (which is added during the assay set up after the addition of stimulants to the cells) resulting in the identification of degranulating cells[257].

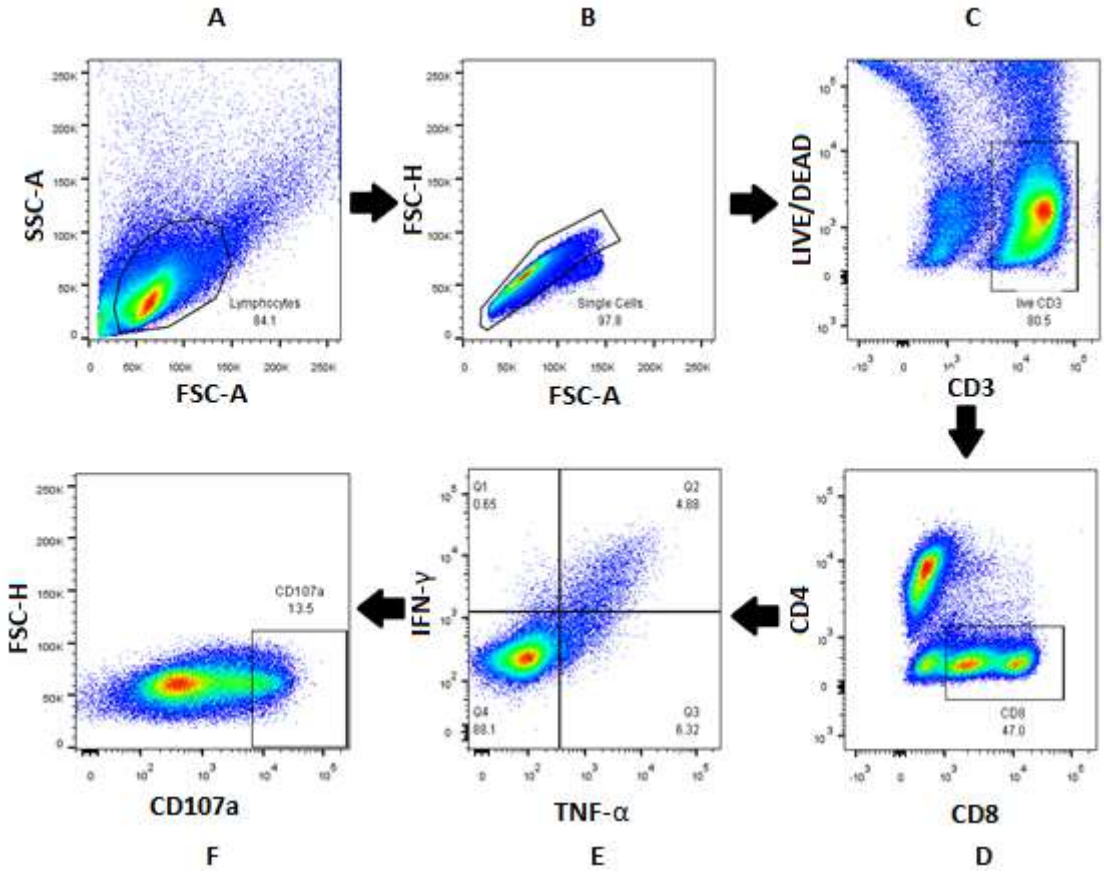


Figure 13. Representative gating strategy used to analyse responding T cells.
Lymphocytes were gated using forward and side scatters (A). Forward scatter area vs height was used to gate on single cells (B). Live T cells were gated as live/dead^{low} CD3^{+} cells (C) and differentiated into CD4 and CD8 subsets (D). Responding T cells were gated by analysing the secretion of IFNγ and TNFα in any combination (E). Finally, degranulating T cells were gated as CD107a^{+} cells (F).

Plasmablast staining

Thawed PBMC were counted and 5x10^{5} cells were plated in a 96 round bottom well plate in R10. Subsequently, cells were incubated for 20 minutes in the dark at room temperature with live/dead (Far red, BD) (diluted 1:1000 in PBS) and stained with labelled antibodies (CD19, CD20, CD38, CD27, details in table 11) for 30 minutes at 2-8°C in a volume of 50µl of PBS. Finally, antibodies were washed away and cells re-suspended in PBS before acquiring them on FACS Verse together with compensation controls. Data analysis was performed using FlowJo with the gating strategy described in figure 14. Forward and side scatter were used to gate on lymphocytes (figure 14A) whereas forward scatter area vs height was used to exclude duplets and gate single

cells only (figure 14B). Live/dead staining was performed to discriminate live from dead cells (figure 14C). The B cell markers CD19 and CD20 were used to discriminate plasmablasts with this cell population being $CD19^{+}CD20^{-}$ (figure 14D). Finally, plasmablasts were gated as $CD27^{high}CD38^{high}$ cells (figure 14E). CD27 is a marker for memory B cells whereas CD38 is a marker of cellular activation and proliferation.

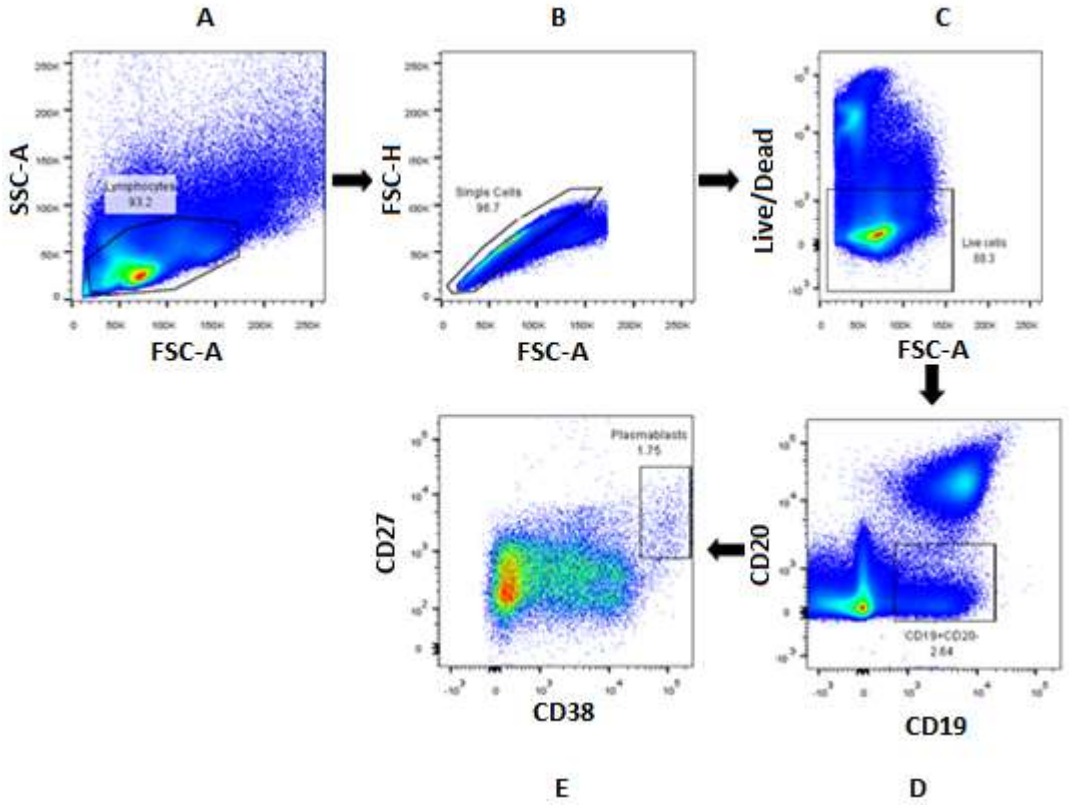


Figure 14. Representative gating strategy used to identify plasmablast cells.
Lymphocytes were gated using forward and side scatters (A). Forward scatter area vs height was used to gate on single cells (B). Live cells were gated as live/dead^{low} cell population (C) and plasmablasts gated as $CD19^{+}CD20^{-}$ (D) and $CD27^{high}CD38^{high}$ (E) cells.

ELISpot

All the reagents used to perform IFNγ-ELISpot were purchased from Mabtech. The day before the assay was carried out, 96 well polyvinylidene difluoride membrane plates were coated with 100µl/well of anti-IFNγ monoclonal antibody (clone 1-D1K) at a final concentration of 10µg/ml in PBS. Subsequently, the plate was wrapped in parafilm and incubated at 2-8°C overnight. The following day, the plate was washed 4 times with 200µl/well of PBS and 200µl of R10 added to each well. The plate was

incubated at 37°C, 5% CO_2 for at least 2 hours. Overnight rested PBMC were washed with R10 and added to a cell density of $2x10^5$ cells/well in a final volume of 50µl of R10. Peptide mini pools or single peptides were diluted at a concentration of 6µg/ml in R10 and 50µl added to wells to achieve a final concentration of 3µg/ml. A volume of 50µl of R10 with 1% DMSO was added to the negative control well whereas 50µl of R10 with Concanavalin A (Sigma) at a concentration of 10µg/ml was added to the positive control wells. Plate was finally incubated at 37°C, 5% CO_2 overnight. The following day, the plate was washed 7 times with 200µl of PBS Tween 20 (0.05%) (PBST) and 50µl of the biotinylated anti-IFNγ antibody (clone 7-B6-1) at a final concentration of 0.5µg/ml (diluted in PBS/0.5% bovine serum albumin (BSA)) was added to each well and the plate incubated at room temperature at dark for 1 hour. Subsequently, the plate was washed 4 times with PBST before adding 50µl of streptavidin conjugated alkaline phosphatase diluted 1:1000 in PBS/0.5% BSA. Plate was incubated for 1 hour at dark at room temperature. Finally, the plate was washed 4 times with PBST and 50µl of filtered 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) added and incubated for 5-15 minutes till spots developed and reaction was stopped by washing the plate with distilled water 4 times. The plate was dried out before reading the spots on an AID ELISpot reader. A response was considered positive if the spot count was at least three times the negative control.

ELISA

IgM and IgG against JEV and DENV were detected by using 4 kits (table 17) according to manufacturer's instructions.

Table 17. ELISA kits used for detection of IgG and IgM specific to JEV and DENV

Virus	Antibody type	Kit	Manufacturer
JEV	IgM	JEV IgM Capture ELISA	National Institute of Virology (Pune)
	IgG	JE Detect IgG ELISA	InBios
DENV	IgM	Panbio Dengue IgM capture ELISA	Alere
	IgG	Panbio dengue IgG indirect ELISA	Alere

Briefly, for JEV IgM, heat inactivated sera were diluted 1:100 and added to the 96-well ELISA plate and incubated for 1 hour at 37°C. At the end of the incubation, samples were washed off and JEV antigen added and incubated for 1 hour at 37°C. Subsequently, an anti-JEV monoclonal antibody was added and incubated for 1 hour followed by 1 hour of incubation in presence of avid-horseradish peroxidase (HRP). Colour was developed by adding 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and stop solution added after 10 minutes. Finally, optical density (OD) was measured at 450nm. Sample readout was based on manufacturer's instructions. A sample was deemed positive if the OD value was higher that one of the negative control by a factor of 3. If the OD value of the sample was exceeding that one of the negative control by a factor between 2 and 3-fold, the sample was considered equivocal whereas if it was less than 2-fold, the sample was considered negative.

For JEV IgG, heat inactivated sera were diluted 1:300 and added to the 96-well ELISA plate pre-coated with JEV recombinant antigen and normal cell antigen. After having washed away the samples, anti-IgG HRP conjugated antibodies were added and colour developed by adding TMB followed by adding stopping solution. OD values were read at 450nm. Sample readout was based on manufacturer's instructions. An immune status ratio was calculated and a value less than 2 was considered negative, a value

between 2 and 5 was considered equivocal whereas a value above 5 was considered positive.

For DENV IgM, heat inactivated sera were diluted 1:100 and added on anti-human IgM pre-coated 96 well ELISA plates. After 1 hour of incubation, antigen-monoclonal antibody conjugated with HRP was added and incubated for another hour. At the end of the incubation, colour was developed by adding TMB and stopped by adding stopping solution. OD values were read at 450nm with a reference filter at 600-650nm. Sample readout and OD ratio was calculated according to manufacturer's instructions. An OD ratio below 0.9 was deemed as negative, whereas and OD ratio between 0.9 and 1.1 was considered equivocal. Finally, an OD ratio above 1.1 was considered positive.

Regarding DENV IgG, heat inactivated sera were diluted 1:100 and added to 96 well ELISA plate and incubated for 30 minutes at 37°C. At the end of the incubation period, anti-human IgG HRP conjugated were added and colour developed by adding TMB followed by adding stopping solution. OD values were read at 450nm with a reference filter at 600-650nm. Sample readout and OD ratio was calculated according to manufacturer's instructions. An OD ratio below 0.9 was deemed as negative, whereas and OD ratio between 0.9 and 1.1 was considered equivocal. Finally, an OD ratio above 1.1 was considered positive.

Statistic

$PRNT_{70}$ values were calculated by probit analysis using SPSS software. A $PRNT_{70}$ value of 1:10 was considered positive. If a volunteer had $PRNT_{70}$ above 1:10 at baseline, a 4-fold increase was considered as response to the vaccine. Statistical analysis were performed with GraphPad Prism. All unpaired non-parametric data were analysed by Mann Whitney U test whereas the paired non-parametric data were analysed by Wilcoxon test. Finally, all correlations were performed with non-parametric data and analysed by Spearman test. P values <0.05 were considered to be statistically significant.

Ethics

The study was conducted according to the principles of the Declaration of Helsinki. Written consent was obtained from all the participants and the protocol approved by the IISc Institutional Human Ethics Committee (ref 5/201), by the Liverpool School

of Tropical Medicine (ref. 10.59) and NIMHANS (NIMH/DO/ETHICS/SUB-COMMETTEE 17th MEETING/2016).

Chapter II: Antibody response to live attenuated Japanese encephalitis vaccine

Introduction

The live attenuated SA14-14-2 Japanese encephalitis (JE) virus (JEV) vaccine (LAJV) is currently the main JE vaccine used in endemic countries. Several studies have already described the safety, immunogenicity and efficiency of this vaccine in children in endemic countries[98, 258].

The gold standard method used to measure neutralising antibodies (NAb) against flaviviruses is by plaque reduction; a 50% plaque reduction neutralisation titre ($PRNT_{50}$) of at least 1:10 is considered protective.

This study was conducted in a country where the 4 serotypes of dengue viruses (DENV) and JEV are co-endemic[259]. As we were interested in analysing the specificity of the NAb against all DENV serotypes as well as JEV, a more stringent PRNT cut-off was used. Therefore we used the 70% plaque reduction titre: the serum dilution able to give a reduction of the number of plaques by 70% in comparison to virus control ($PRNT_{70}$). Furthermore, antibodies that better neutralise DENV grown on C6/36 rather than dendritic cell derived virions have been described[183]. This difference is due to a diverse efficiency in cutting the pr fragment during the maturation process resulting in different prM content in DENV particles grown on insect vs dendritic cells with the latter showing a lower percentage of prM. $PRNT_{50}$ includes the neutralisation effect of these antibodies and therefore can overestimate the neutralisation potential of the antibody titre. However, virus derived from C6/36 cells is thought to represent better the infecting virus derived from a mosquito[260]. As the viruses used in this study were grown on C6/36, use of $PRNT_{70}$ helped to exclude this overestimation.

A titre of at least 1:10 of $PRNT_{70}$ measured following vaccination was used to discriminate responders and non responders. As previously, if a volunteer was seropositive at baseline ($PRNT_{70}$ of 1:10 or above), a 4-fold increase in the NAb titre following vaccination was used to define seroconversion[81,158].

JEV and DENV co-circulate in many different countries in South and South East-Asia[259]. Cross-reactivity between these two flaviviruses raises important diagnostic and clinical concerns. Due to the importance of pre-existing anti-DENV antibodies in severe dengue, the role of antibodies in dengue and more generally in flaviviruses has been studied extensively. Flavivirus cross-reactive antibodies bind to conserved regions of envelope (E) and pre-membrane (prM) proteins[181]. An important target of

these cross-reactive antibodies is the fusion loop (FL): a portion of the domain 2 of the envelope protein which contains a hydrophobic region that is important for the fusion of the virus with the endosomal membrane following a drop in pH. This step is critical for the viral capsid and genome to enter the cytoplasm of the host cell. Despite the important role of this region in the infectious cycle, antibodies against the FL are poorly neutralising. In addition, anti-FL antibodies promote antibody dependent enhancement (ADE) increasing infectivity of DENV to Fc receptor bearing cells[261,182]. More recently, anti-FL antibodies raised following DENV infection were also described in a mechanism of Zika virus (ZIKV) enhancement *in vitro* and *in vivo*[262].

Another well studied target of antibodies is prM. During the process of viral replication inside the host cells, the prM structural protein is cleaved by furin making a mature infectious viral particle. However, this process is inefficient resulting in the production of a mixture of mature, immature and partially mature viral particles. Antibodies targeting the prM can promote ADE allowing immature non-infectious virus particles to infect Fc receptors bearing cells.

As a large component of the antibody responses to flaviviruses consists of non/poorly neutralising antibodies[263], NAb are preferentially used to determine seroconversion in vaccine studies.

The antibody response during a secondary heterologous DENV infection is also characterized by the phenomenon of original antigenic sin (OAS)[195,264]. This phenomenon was also described for the T cell response in DENV infection[202]. The clinical effect of OAS in the antibody response is still not clear. There is some evidence, however, of OAS, or at least cross-reactivity in the T cell response being protective[195,209,210,264].

The work presented in this chapter will focus on the specificity of the antibody response raised following JE vaccination with particular reference to the phenomenon of OAS. Previous studies have shown low immunogenicity of LAJV in India[81,102]. Vaccination with LAJV was used as a model to study the specificity of the antibody response in a secondary flavivirus infection. This model was used to test the hypothesis that participants with previous exposure to DENV will show OAS by mounting a higher NAb response against DENV than JEV following LAJV vaccination possibly explaining the low immunogenicity of the vaccine. The main strength of this project

was the possibility to study the samples obtained just before the vaccine administration and follow the response at different time points after vaccination.

LAJV was administrated to a total of 17 volunteers although one of them withdrew after a week and was excluded for this study. PRNT were measured in serum samples obtained at week 0, 4 and 8. In order to measure binding antibody (as opposed to neutralisation), IgM and IgG ELISAs for JEV and DENV were also performed for samples collected at week 0 and week 4. Finally, plasmablast populations were measured at week 0, 1 and 2 if cells were available.

Although the serum samples collected before vaccination were available from all the participants, one participant was missing a sample at the week 4 time point, and 2 subjects had sample missing at the week 8 time point. All subjects had at least one post vaccine serum sample available.

Results

NAb response specific to JEV following JE vaccination

One participant (015c3k1) was JEV seropositive by $PRNT_{70}$ before vaccination (reciprocal NAb titre of 13). Four weeks after vaccination only 3 participants (18.75%) seroconverted (seroconversion was determined by a titre of at least 1:10 of $PRNT_{70}$ measured following vaccination or at least a 4-fold increase in participants seropositive at baseline) whereas the only participant JEV seropositive at baseline showed an increase of NAb titre of 3.9 fold, therefore this participant was considered as non-responder (table 18).

Eight weeks after vaccination, JEV $PRNT_{70}$ of 1:10 or above were observed in only 2 participants, including the participant seropositive at week 0. However, it is important to note that 2 out of 3 participants who seroconverted at week 4 no longer showed $PRNT_{70}$ 1:10 or above by week 8 (table 18).

Table 18 Reciprocal NAb titre against JEV measured by $PRNT_{70}$

JEV NAb					
Participant ID	**Week 0**	**Week 4**	**Week 8**	**Seroprotected[a] at any time point**	**Vaccine Responder[b]**
005c3k1	0	10	12	Yes	Yes
001c1k1	0	37	9	Yes	Yes
023c1k1	2	22	9	Yes	Yes
015c3k1	13	51	18	Yes	No
023c3k1	0	2	3	No	No
020c1k1	0	0	3	No	No
008c3k1	0	0	0	No	No
019c3k1	0	0	0	No	No
007c1k1	0	0	NA	No	No
017c1k1	0	0	0	No	No
022c3k1	1	0	0	No	No
018c1k1	0	0	0	No	No
012c3k1	2	5	6	No	No
020c3k1	0	0	0	No	No
004c1k1	0	0	NA	No	No
010c3k1	3	NA	7	No	No

[a] Seroprotection is defined as any participant with $PRNT_{70} \geq 1:10$.

[b] Seroconversion (vaccine responder) is defined as $PRNT_{70} \geq 1:10$ measured following vaccination or at least a 4-fold increase in titre in participants seropositive at baseline ($PRNT_{70}$ 1:10 or above).

Overall, 3 out of 16 participants (18.75%) responded to the vaccine with a peak response observed at week 4 and a reciprocal geometric mean titre (GMT) of 20. Eight weeks after vaccination, a drop of the JEV titre was observed with a reciprocal GMT of 11 and $PRNT_{70} \geq 1:10$ detected in only 2 participants (figure 15).

These data demonstrated that LAJV was not very immunogenic for NAb response in the population studied. Additionally, in terms of magnitude of the antibody response, a low level of NAb was detected in all the responders.

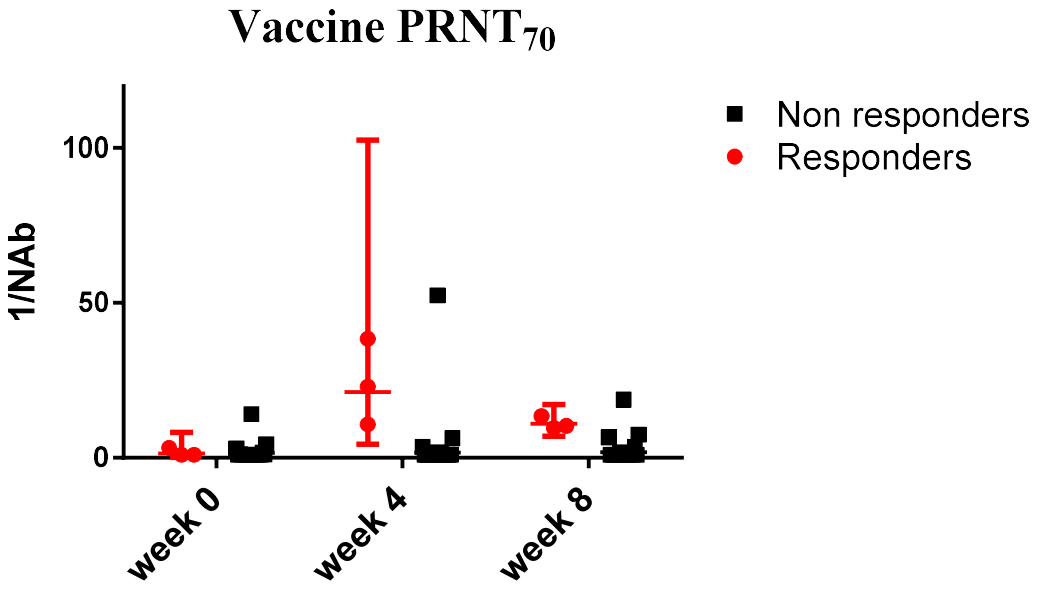


Figure 15. NAb to JEV following LAJV administration.

Reciprocal JEV $PRNT_{70}$ measured at week 0, 4 and 8 in responders vs non responders following LAJV vaccination. Lines and error bars indicate the geometric mean and the 95% confidence interval. One participant who had a reciprocal $PRNT_{70}$ titre of 51 at week 4 was included in the non responders group as it was seropositive at baseline and showed a 3.9-fold change increase following vaccination.

NAb response to DENV following JE vaccination

To test the hypothesis that vaccination with the LAJV could have increased the titre of cross-reactive NAb against one or more DENV serotypes, $PRNT_{70}$ for each DENV serotype were performed at baseline and 4 and 8 weeks after vaccination. Overall, NAb against all DENV serotypes were detected (table 19).

Table 19. Prevalence of NAb against the 4 DENV serotypes

DENV serotype	Participants with DENV NAb at baseline	Participants with DENV NAb at any time point
DENV1	10 (62.5%)	11 (68.75%)
DENV2	11 (68.75%)	11 (68.75%)
DENV3	8 (50%)	10 (62.5%)
DENV4	5 (31.25%)	6 (37.5%)

DENV1

NAb against DENV1 was detected in a total of 11 participants (at any time point). Among them, one participant (019c3k1) who had $PRNT_{70}$ <10 at week 0 seroconverted at week 4 (1:31) and week 8 (1:19). The reciprocal GMT of NAb against DENV1 at baseline in the 11 participants who were DENV1 seropositive (at any time point) was 110.7. No increase in NAb titre against DENV1 among the DENV1 seropositive (at any time point) participants was observed following JEV vaccination with a reciprocal GMT of 103.1 at week 4 and 103.5 at week 8 (figure 16). One volunteer (022c3k1) had a reciprocal NAb titre of 10 at baseline that dropped to below 10 at week 4 and 8.

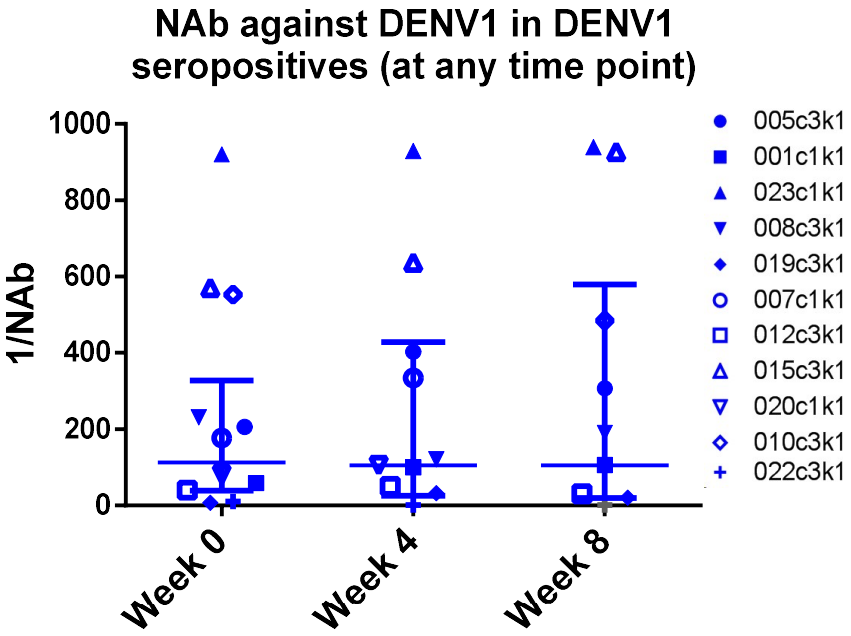


Figure 16. NAb against DENV1 following LAJV administration.

Reciprocal of DENV1 NAb titre measured by $PRNT_{70}$ in DENV1 seropositives (at any time point) (n=11) participants measured at week 0, 4 and 8 following LAJV vaccination. Lines and error bars indicate the geometric mean and the 95% confidence interval.

Table 20. Reciprocal NAb titre against DENV1 measured by $PRNT_{70}$

DENV1 NAb titre				
Participant ID	**Week 0**	**Week 4**	**Week 8**	**Vaccine Responder**
005c3k1	205	402	306	Yes
001c1k1	58	99	106	Yes
023c1k1	919	928	939	Yes
015c3k1	570	636	926	No
023c3k1	0	0	0	No
020c1k1	81	104	115	No
008c3k1	230	120	190	No
019c3k1	6	31	19	No
007c1k1	176	333	NA	No
017c1k1	0	0	0	No
022c3k1	10	0	0	No
018c1k1	0	0	0	No
012c3k1	39	49	30	No
020c3k1	0	0	0	No
004c1k1	0	0	NA	No
010c3k1	552	NA	484	No

DENV2

Anti-DENV2 NAb were observed in 11 participants with a reciprocal GMT of 284.2, 291.6 and 324.2 at week 0, 4 and 8 respectively. Therefore, no increase in NAb titre was observed following JE vaccination among the DENV2 seropositive participants (figure 17 and table 21).

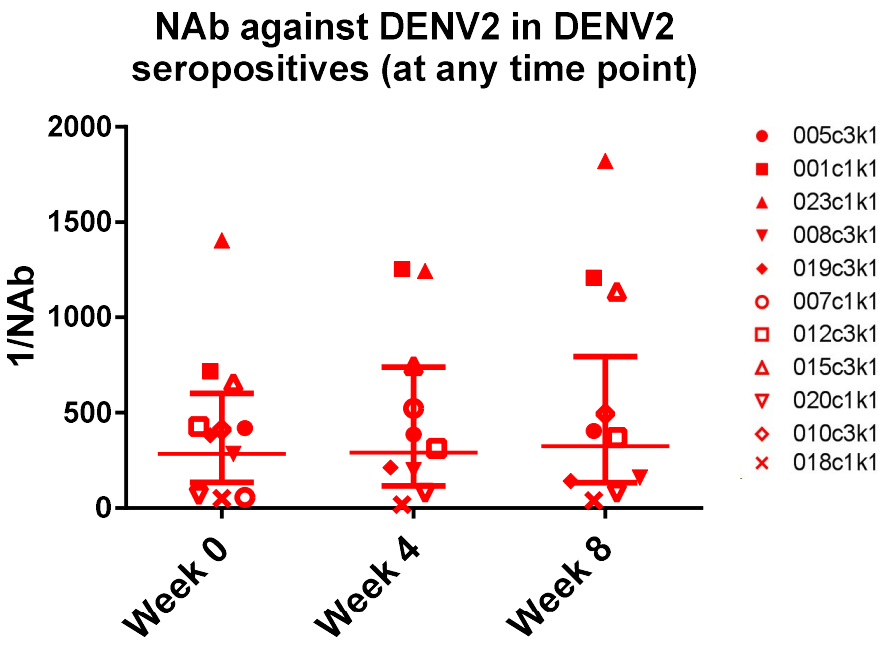


Figure 17. NAb against DENV2 following LAJV administration

Reciprocal of DENV2 NAb titre measured by $PRNT_{70}$ in DENV2 seropositives (at any time point) (n=11) participants measured at week 0, 4 and 8 following LAJV vaccination. Lines and error bars indicate the geometric mean and the 95% confidence interval.

Table 21. Reciprocal NAb titre against DENV2 measured by $PRNT_{70}$

DENV2 NAb titre				
Participant ID	**Week 0**	**Week 4**	**Week 8**	**Vaccine Responder**
005c3k1	420	386	404	Yes
001c1k1	717	1254	1207	Yes
023c1k1	1405	1244	1820	Yes
015c3k1	657	747	1138	No
023c3k1	8	4	2	No
020c1k1	66	78	78	No
008c3k1	285	200	159	No
019c3k1	380	212	142	No
007c1k1	55	522	NA	No
017c1k1	0	0	0	No
022c3k1	1	0	2	No
018c1k1	50	18	40	No
012c3k1	428	313	367	No
020c3k1	0	0	0	No
004c1k1	0	0	NA	No
010c3k1	410	NA	494	No

DENV3

A total of 10 participants showed NAb against DENV3 during the course of the study with a reciprocal GMT of 32.7 at baseline. Two of them (019c3k1 and 007c1k1) were seronegative at week 0 and showed an increase of NAb following LAJV vaccination only at week 4. The reciprocal GMT calculated among these 10 participants against DENV3 was 57.2 at week 4 and 57 at week 8. Overall, a median of 1.1 and 1.3 fold increase of the NAb titre over baseline was measured for week 4 and week 8 respectively (figure 18).

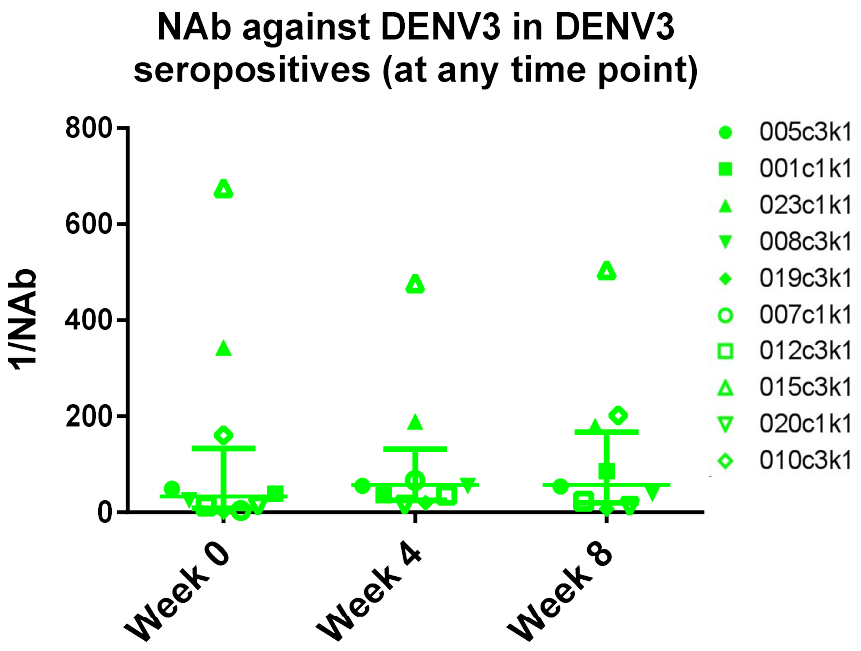


Figure 18. NAb against DENV3 following LAJV administration.

Reciprocal of DENV3 NAb titre measured by $PRNT_{70}$ in DENV3 seropositives (at any time point) (n=10) participants measured at week 0, 4 and 8 following LAJV vaccination. Lines and error bars indicate the geometric mean and the 95% confidence interval.

Table 22. Reciprocal NAb titre against DENV3 measured by $PRNT_{70}$.

DENV3 NAb titre				
Participant ID	**Week 0**	**Week 4**	**Week 8**	**Vaccine Responder**
005c3k1	49	55	53	Yes
001c1k1	39	35	85	Yes
023c1k1	343	189	179	Yes
015c3k1	675	478	505	No
023c3k1	0	0	0	No
020c1k1	13	15	13	No
008c3k1	25	55	39	No
019c3k1	1	20	7	No
007c1k1	4	66	NA	No
017c1k1	0	0	1	No
022c3k1	0	0	0	No
018c1k1	0	0	0	No
012c3k1	13	35	23	No
020c3k1	4	0	0	No
004c1k1	0	0	NA	No
010c3k1	160	NA	202	No

DENV 4

NAb against DENV4 were less prevalent, being detected (at any time point) in only 6 participants. The reciprocal GMT of NAb against DENV4 at baseline in DENV4 seropositives (at any time point) was 79. Although not statistically significant an increase in GMT was observed at week 4 and 8 with a reciprocal GMT of respectively 99.2 and 159.3 (figure 19). As observed for DENV1 and DENV3, one participant (005c3k1) who was seronegative at baseline showed NAb against DENV4 at week 4 (1:37) and week 8 (1:54). Overall, the data regarding the NAb titre against DENV4 from these 6 participants indicated a 1 and 2.2 median fold increase at week 4 and 8 respectively.

Finally, four participants were seronegative at baseline for all the 4 DENV serotypes and remained seronegative throughout the whole study period.

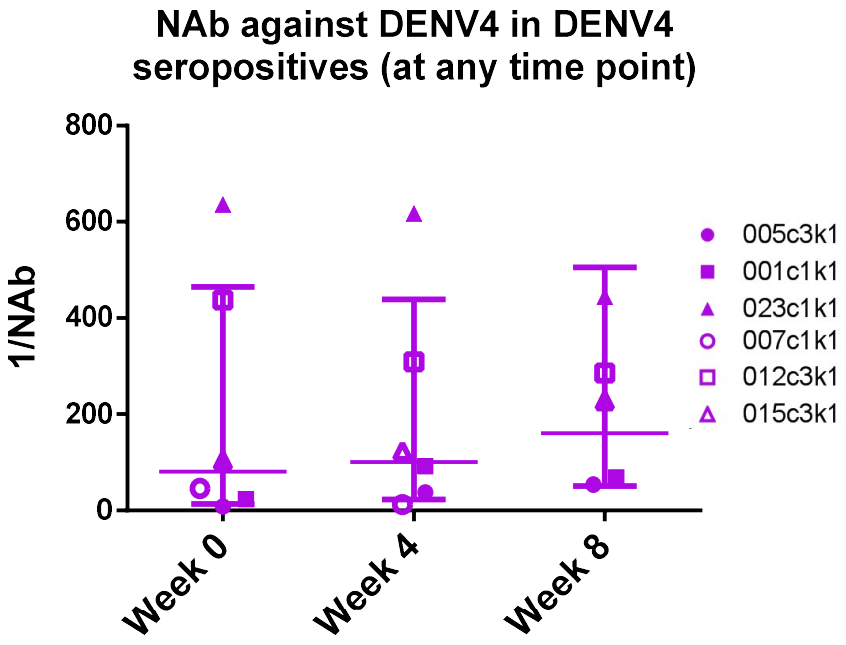


Figure 19. NAb against DENV4 foloowing LAJV administration.

Reciprocal of DENV4 NAb titre measured by $PRNT_{70}$ in DENV4 seropositives (at any time point) (n=6) participants measured at week 0, 4 and 8 following LAJV vaccination. Lines and error bars indicate the geometric mean and the 95% confidence interval.

Table 23. Reciprocal NAb titre against DENV4 measured by $PRNT_{70}$

DENV 4 NAb titre				
Participant ID	**Week 0**	**Week 4**	**Week 8**	**Vaccine Responder**
005c3k1	9	37	54	Yes
001c1k1	23	92	68	Yes
023c1k1	636	617	444	Yes
015c3k1	103	123	228	No
023c3k1	0	0	0	No
020c1k1	2	8	5	No
008c3k1	0	0	0	No
019c3k1	0	0	0	No
007c1k1	45	13	NA	No
017c1k1	0	0	0	No
022c3k1	2	0	0	No
018c1k1	0	0	2	No
012c3k1	438	309	286	No
020c3k1	0	0	0	No
004c1k1	0	0	NA	No
010c3k1	1	NA	3	No

Effect of DENV NAb on immunogenicity of LAJV

Previously, Chan *et al.* demonstrated that pre-existing NAb against inactivated JE vaccine increased the immunogenicity of yellow fever vaccination[243]. To test the hypothesis that previous DENV exposure may increase the immunogenicity of the LAJV, the NAb response to LAJV was analysed by DENV serostatus at baseline. Interestingly, for all DENV serotypes, the GMT in those who responded to JE vaccine was higher than those who did not. However, this difference was statistically significant (p value <0.05, Mann-Whitney test) only for DENV2 (figure 20A, table 24 and 25). To exclude the possibility that is effect may be driven by DENV seronegative participants who are only present in the group which did not respond to LAJV, they were excluded in a second analysis. This confirmed that higher DENV2 NAb was

observed in the responder group (p value <0.05, Mann-Whitney test) (figure 20B, table 24 and 25).

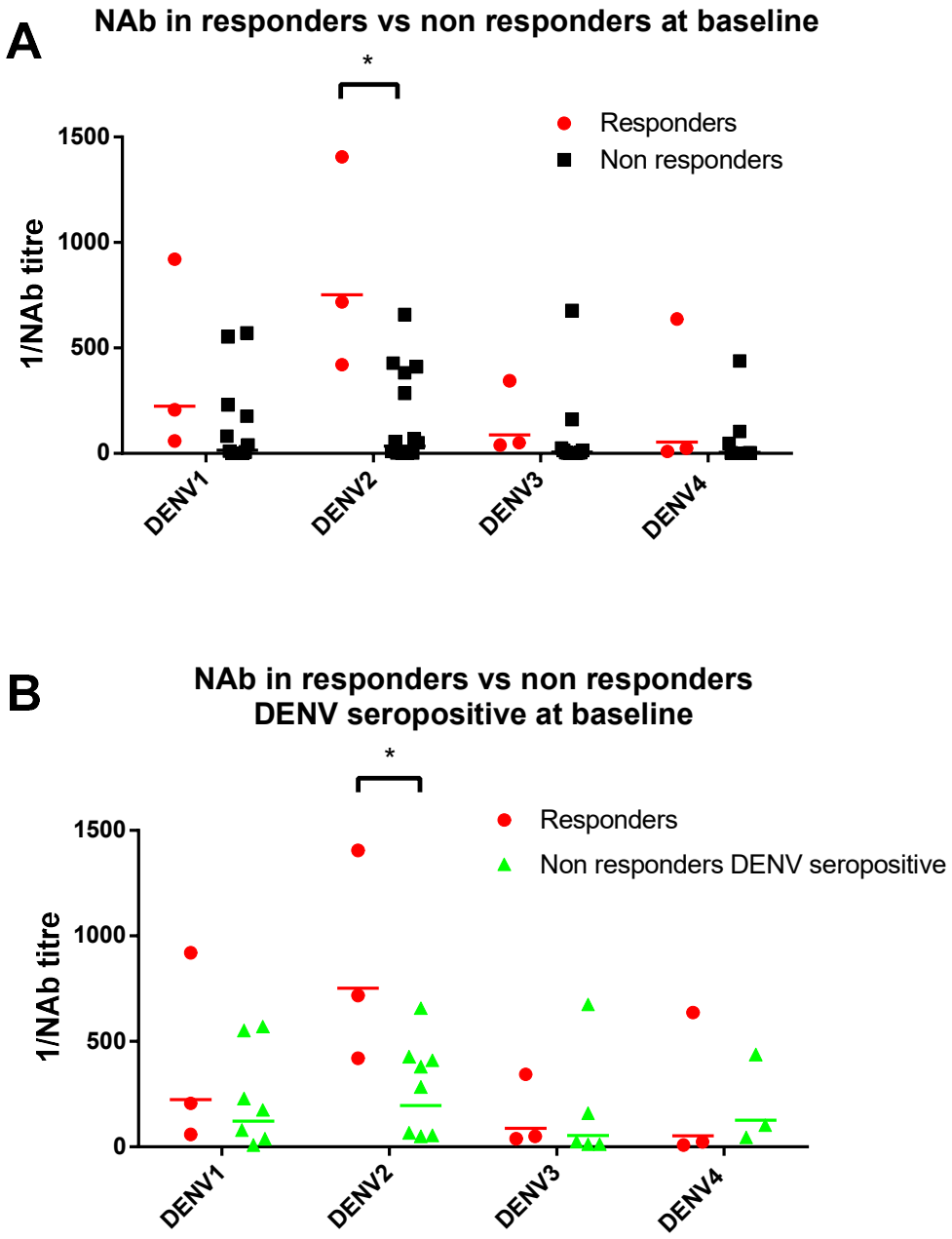


Figure 20. DENV NAb at baseline in responders vs non responders to LAJV.

Reciprocal $PRNT_{70}$ against DENV1-4 measured in responders vs non responders to the LAJV (A) and responders vs non responders DENV seropositive at baseline to the LAJV (B) at week 0. Asterisks indicate a p value <0.05 analysed with Mann-Whitney test. Lines indicate GMT.

Although the number of participants was small, this analysis suggested that DENV serostatus at baseline has the potential to influence the JE vaccine immunogenicity. The fact that statistical significance was observed only against DENV2 may be due to the higher prevalence and NAb titre of DENV2 in Karnataka state[265].

Following LAJV, no change in NAb titre against DENV1, DENV2 and DENV3 was seen in the responder group. On the contrary, NAb titres against DENV4 showed a median increase of 3.9 fold at week 4 and 2.9 fold at week 8. No overall change was observed in the participants who did not respond to the vaccine although a 2.5 fold increase was observed for DENV3 when DENV seronegative individuals were excluded from the analysis (figure 21 and table 24 and 24). Although these data confirmed that some individuals developed DENV NAb following LAJV, the small sample size precluded meaningful statistical testing.

In summary, these data provided no evidence that previous DENV infection interferes with JE vaccination. On the contrary, higher DENV NAb at baseline may play a role in increasing immunogenicity of the vaccine.

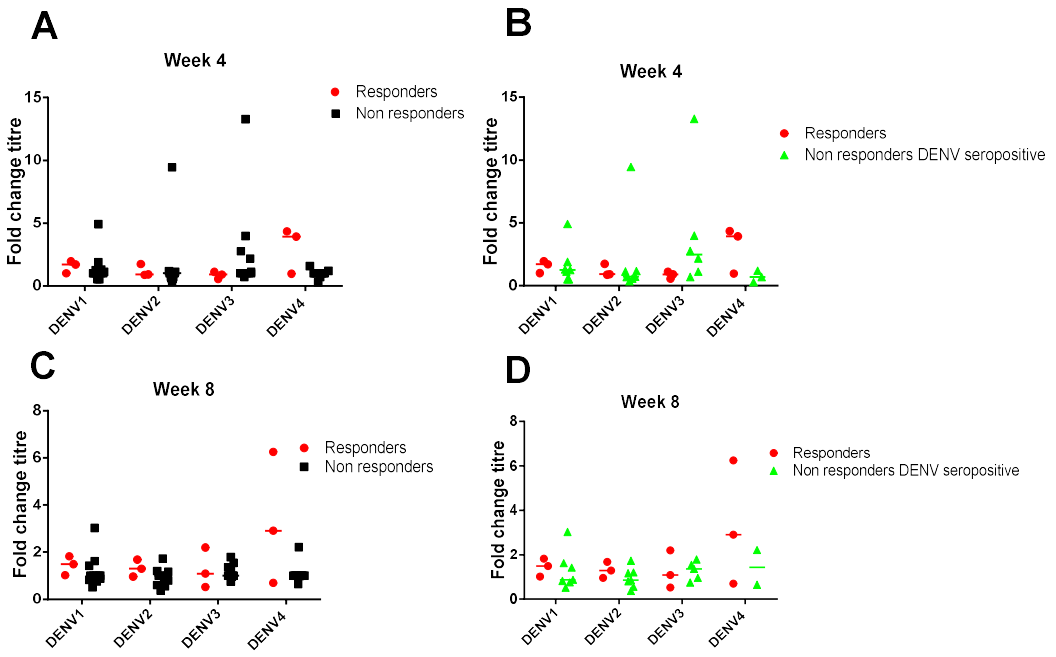


Figure 21. Fold change DENV NAb titre following LAJV vaccination.

Fold change NAb titre against DENV1-4 at week 4 (A and B) and 8 (C and D) among responders vs non responders (A and C) and responders vs non responders DENV seropositive (B and D) following LAJV. Lines indicate median values.

Table 24. Summary table of GMT of all viruses at all time points stratified per vaccine responsiveness

	Time point	NAb GMT				
		JEV	DENV1	DENV2	DENV3	DENV4
Responders	Week 0	<10	223	751	87	52
	Week 4	21	334	844	72	129
	Week 8	10	312	961	94	118
Non responders	Week 0	<10	15	33	<10	<10
	Week 4	<10	10	24	<10	<10
	Week 8	<10	11	33	<10	<10

Table 25. Summary table of GMT of all viruses at all time points stratified per vaccine responsiveness and DENV serological status

	Time point	NAb GMT				
		JEV	DENV1	DENV2	DENV3	DENV4
Responders	Week 0	<10	223	751	87	52
	Week 4	21	334	844	72	129
	Week 8	10	312	961	94	118
Non responders DENV seropositive	Week 0	<10	86	198	24	128
	Week 4	<10	63	186	52	79
	Week 8	<10	60	240	59	255
Non responders DENV seronegative	Week 0	<10	<10	<10	<10	<10
	Week 4	<10	<10	<10	<10	<10
	Week 8	<10	<10	<10	<10	<10

ELISA IgG and IgM

PRNT allows the detection of NAb only. Most of the participants showed no change in NAb titres after LAJV. We hypothesised that one reason for this may be that non-neutralising antibody is made following vaccination. In order to test this hypothesis, ELISA for IgG and IgM specific to JEV and DENV on the serum samples collected at week 0 and 4 were performed.

Following a viral infection the first class of antibodies produced are IgM. Detection of JEV specific IgM may therefore identify participants who did not develop NAb but responded to the LAJV by making non-neutralising antibody of IgM subclass. One participant (007c1k1) was JEV IgM positive before vaccination and at week 4. As this participant was dengue IgM negative, cross-reactivity with dengue IgM is less likely. This result could indicate a possible recent asymptomatic infection with wild type JEV, or a false positive. This participant was an adult resident in urban Bangalore in good health, making recent JEV infection very unlikely. Overall, five participants tested positive at week 4 to JEV IgM, including all the responders by $PRNT_{70}$ (table 26).

When analysing the total level of JEV IgM a statistically significant higher OD ratio was observed following LAJV vaccination (p value <0.05, Wilcoxon test) (figure 22).

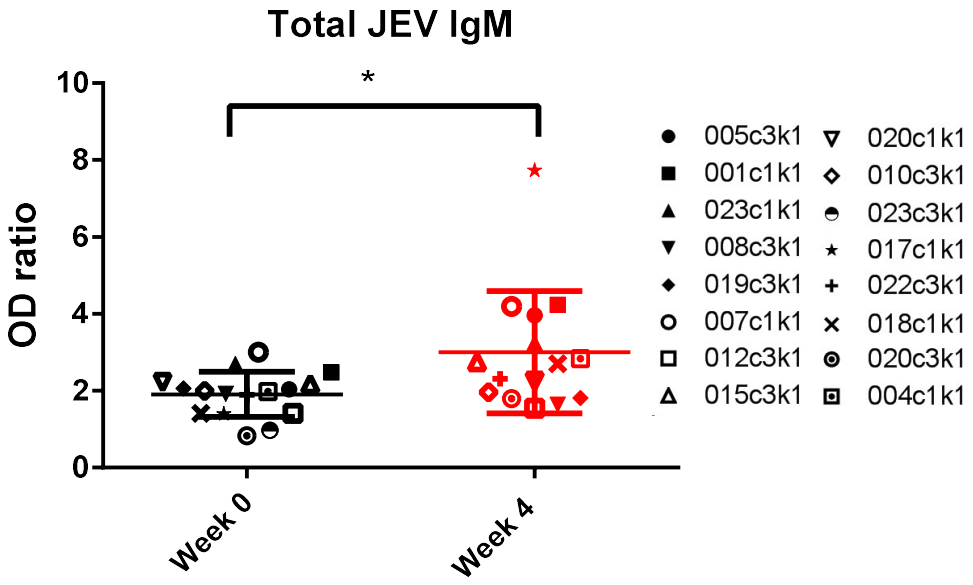


Figure 22. Total JEV IgM following LAJV administration.

JEV IgM OD ratio before and after vaccination in all participants. Asterisks indicate p value <0.05 by Wilcoxon test. Error bars represent the median and interquartile range.

Table 26. OD ratio IgM JEV specific measured at baseline and 4 weeks after vaccination.

JEV IgM ELISA					
Participant ID	**Week 0**		**Week 4**		**Vaccine responder by $PRNT_{70}$**
	OD ratio	**ELISA readout**	**OD ratio**	**ELISA readout**	
005c3k1	2.03814	Equivocal	3.9661	Positive	Yes
001c1k1	2.47881	Equivocal	4.24153	Positive	Yes
023c1k1	2.69492	Equivocal	3.25424	Positive	Yes
015c3k1	2.182203	Equivocal	2.754237	Equivocal	No
023c3k1	0.97458	Negative	1.9661	Negative	No
020c1k1	2.22458	Equivocal	2.25424	Equivocal	No
008c3k1	1.923729	Negative	1.639831	Negative	No
019c3k1	2.05932	Equivocal	1.80932	Negative	No
007c1k1	3	Positive	4.19915	Positive	No
017c1k1	1.39831	Negative	7.72881	Positive	No
022c3k1	1.89831	Negative	2.31356	Equivocal	No
018c1k1	1.41525	Negative	2.70339	Equivocal	No
012c3k1	1.41525	Negative	1.54237	Negative	No
020c3k1	0.83051	Negative	1.79237	Negative	No
004c1k1	1.98305	Negative	2.83898	Equivocal	No
010c3k1	1.99576	Negative	NA	NA	No

When stratifying according to the response to the vaccine by $PRNT_{70}$, a higher OD ratio was observed at week 4 in both groups. However, this increase was statistically significant only for the participants who did not make NAb (p value <0.05, measured by Wilcoxon test) (Figure 23). This result remained significant even after excluding the participant with the OD ratio of 7.7 at week 4. Overall, these data may indicate that most participants responded to the vaccine by producing antibodies that did not neutralise, or did not neutralise very well. In particular, one participant, 017c1k1,

showed clear evidence of seroconversion, with a change in OD ratio of 1.4 pre vaccine to 7.7 afterwards.

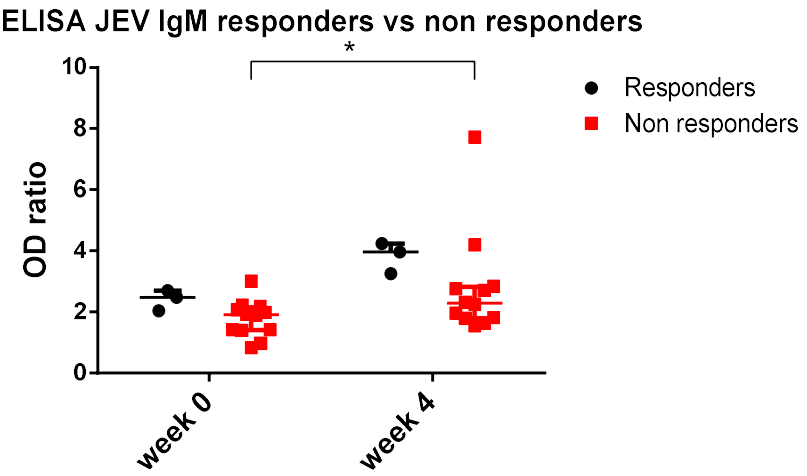


Figure 23. JEV IgM among responders and non responders (by NAb) to the LAJV.

OD ratio of IgM specific to JEV in vaccine responders and non responders to the LAJV by $PRNT_{70}$ measured at baseline and 4 weeks after vaccination. Asterisks indicate p value <0.05 analysed by Wilcoxon test. Error bars represent median and interquartile range.

As some participants showed increases in DENV specific NAb after LAJV to exclude the possibility that this was due to an asymptomatic DENV infection, DENV specific IgM were measured by ELISA at week 0 and 4. DENV IgM was negative at both time points in all the participants. These data indicated that a DENV infection of the participants during the study period was unlikely (table 27).

Table 27. OD ratio IgM DENV specific measured at baseline and 4 weeks after vaccination.

DENV IgM ELISA					
Participant ID	**Week 0**		**Week 4**		**Vaccine responder by $PRNT_{70}$**
	OD ratio	**ELISA readout**	**OD ratio**	**ELISA readout**	
005c3k1	0.29346	Negative	0.28368	Negative	Yes
001c1k1	0.22079	Negative	0.19145	Negative	Yes
023c1k1	0.40525	Negative	0.37311	Negative	Yes
015c3k1	0.19704	Negative	0.17887	Negative	No
023c3k1	0.05031	Negative	0.02655	Negative	No
020c1k1	0.600894	Negative	0.463946	Negative	No
008c3k1	0.11878	Negative	0.1062	Negative	No
019c3k1	0.15931	Negative	0.20263	Negative	No
007c1k1	0.12856	Negative	0.19843	Negative	No
017c1k1	0.11319	Negative	0.1607	Negative	No
022c3k1	0.05729	Negative	0.07965	Negative	No
018c1k1	0.91951	Equivocal	0.92091	Equivocal	No
012c3k1	0.15092	Negative	0.17188	Negative	No
020c3k1	0.09782	Negative	0.07686	Negative	No
004c1k1	0.173281	Negative	0.017188	Negative	No
010c3k1	0.12437	Negative	NA	NA	No

Following the initial IgM response, B cells undergo a class switching and affinity maturation processes which result in an antibody response dominated by IgG. Therefore, JEV specific IgG levels were measured at baseline and week 4 following LAJV administration. Detection of JEV specific IgG may identify participants who did not develop NAb but responded to the LAJV by making non-neutralising antibody of IgG subclass. Two participants had a positive result in the JEV IgG ELISA before vaccination. After LAJV administration, JEV IgG ELISA OD ratios did not increase; 4 weeks after vaccination only 1 participant was positive to JEV IgG (figure 24 and table 28).

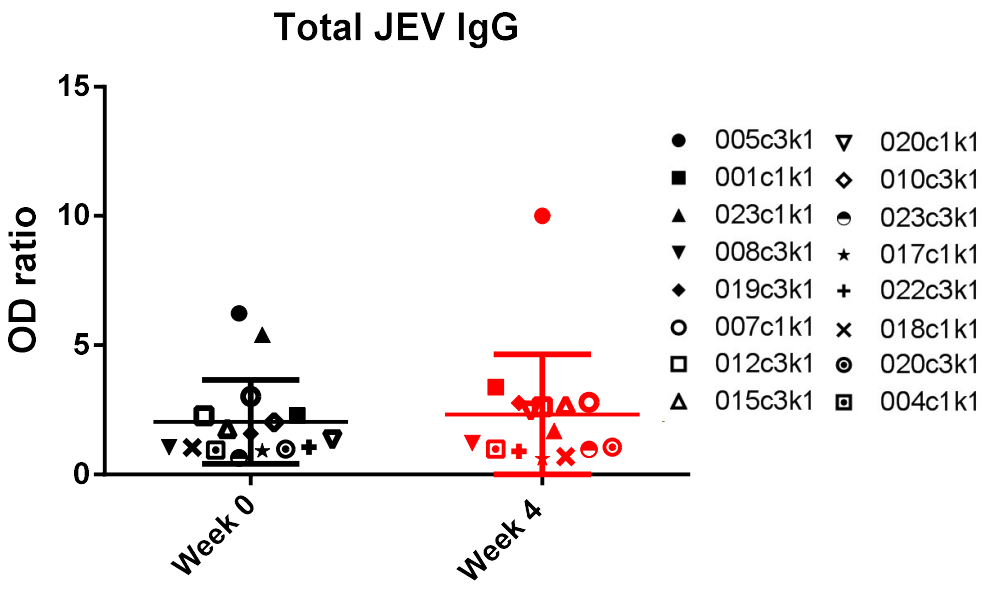


Figure 24. Total JEV IgG following LAJV administration.

JEV IgG OD ratio before and after vaccination in all participants. Error bars represent the median and interquartile range.

Subdividing by response to LAJV by $PRNT_{70}$, there was no further evidence of an IgG response to LAJV in either those who made neutralising antibody responses or those who did not. Higher JEV IgG was observed among responders vs non responders (by $PRNT_{70}$) which was statistically significant at baseline only ($p<0.05$, Mann-Whitney U test) (figure 25). The observation that higher OD ratios were observed at baseline in the participants who mounted NAb responses following LAJV, and that the 2 volunteers JEV IgG positive at baseline were JEV seronegative and DENV seropositive at baseline by $PRNT_{70}$, suggested the possibility of cross-reactivity between JEV and DENV detected by this assay.

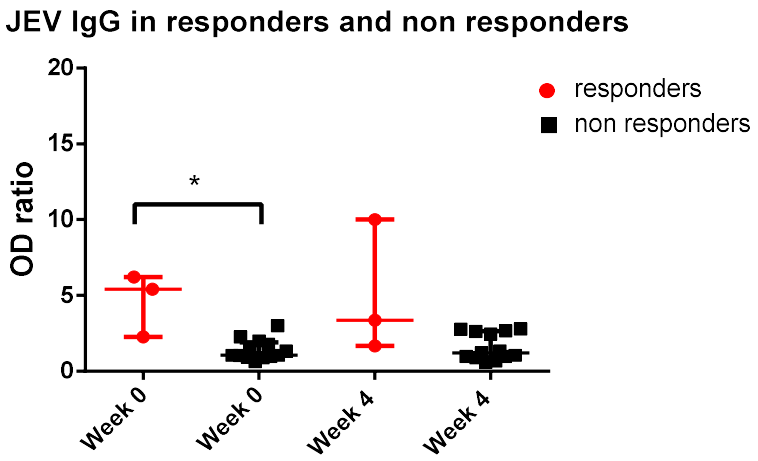


Figure 25. JEV IgG among responders and non responders (by NAb) to the LAJV.

OD ratio of IgG specific to JEV in vaccine responders and non responders to the LAJV by $PRNT_{70}$ measured at baseline and 4 weeks after vaccination. Asterisks indicate p value <0.05 analysed by Mann-Whitney U test. Error bars represent median and interquartile range.

Table 28. OD ratio of JEV specific IgG detected at baseline and 4 weeks after vaccination

JEV IgG ELISA					
Participant ID	**Week 0**		**Week 4**		**Vaccine responder by $PRNT_{70}$**
	OD ratio	**ELISA readout**	**OD ratio**	**ELISA readout**	
005c3k1	6.23214	Positive	10	Positive	Yes
001c1k1	2.27941	Equivocal	3.37879	Equivocal	Yes
023c1k1	5.40741	Positive	1.67797	Negative	Yes
015c3k1	1.78689	Negative	2.68254	Equivocal	No
023c3k1	0.64894	Negative	0.96825	Negative	No
020c1k1	1.34545	Negative	2.46	Equivocal	No
008c3k1	1.05455	Negative	1.21311	Negative	No
019c3k1	1.58065	Negative	2.77419	Equivocal	No
007c1k1	3.0137	Equivocal	2.7973	Equivocal	No
017c1k1	0.90476	Negative	0.60185	Negative	No
022c3k1	1.05085	Negative	0.89231	Negative	No
018c1k1	1.04839	Negative	0.69318	Negative	No
012c3k1	2.2716	Equivocal	2.62121	Equivocal	No
020c3k1	0.98214	Negative	1.05172	Negative	No
004c1k1	0.94286	Negative	0.98551	Negative	No
010c3k1	2.01389	Equivocal	NA	NA	No

To test the hypothesis that the JEV IgG assay may detect cross-reactive antibodies, JEV IgG data were analysed according to DENV serological status. The JEV IgG OD ratio was significantly lower in DENV seronegative compared with seropositive participants (by $PRNT_{70}$) at baseline (Mann-Whitney test, $p<0.05$) and 4 weeks after vaccination (Mann-Whitney test, $p<0.05$) (figure 26). Furthermore, only a modest correlation was found among JEV $PRNT_{70}$ and JEV IgG ELISA for the data at week 4 (Spearman, $r=0.5288$, $p<0.05$) (figure 27). On the contrary, no correlation was found for the data at week 0 (Spearman, $r=0.2171$, $p=0.4172$) (figure 27). Finally, a better correlation was observed between JEV IgG and DENV NAb titre, irrespective of

serotype, compared with JEV $PRNT_{70}$ (figure 28). Altogether these data strongly suggest that this JEV IgG assay detects antibodies that are cross-reactive with DENV.

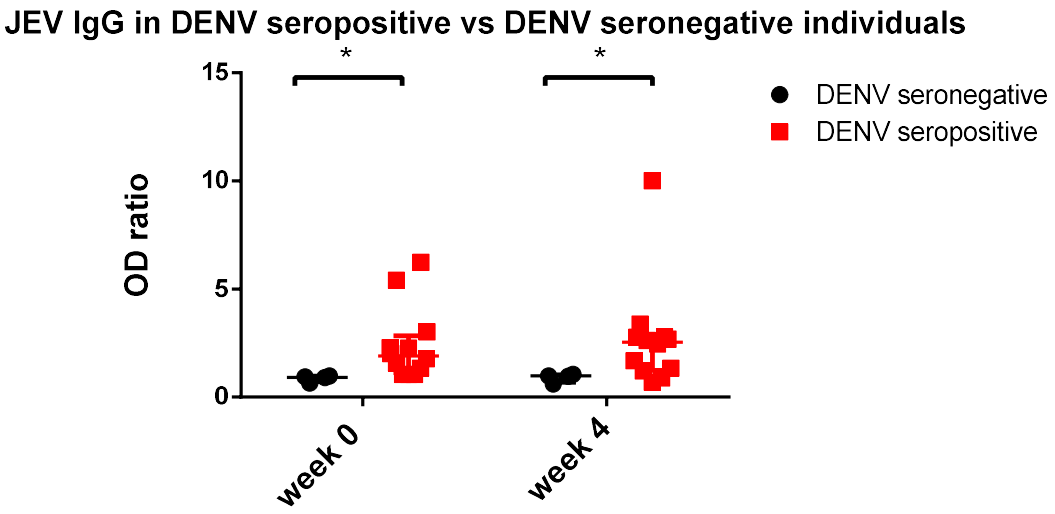


Figure 26. JEV IgG following LAJV in participants DENV seropositive vs seronegative.

JEV IgG OD ratio in DENV seronegative and DENV seropositive (by $PRNT_{70}$) participants at week 0 and 4 following vaccination. Asterisks indicate p value <0.05 by Mann-Whitney U test. Error bars represent the median and interquartile range.

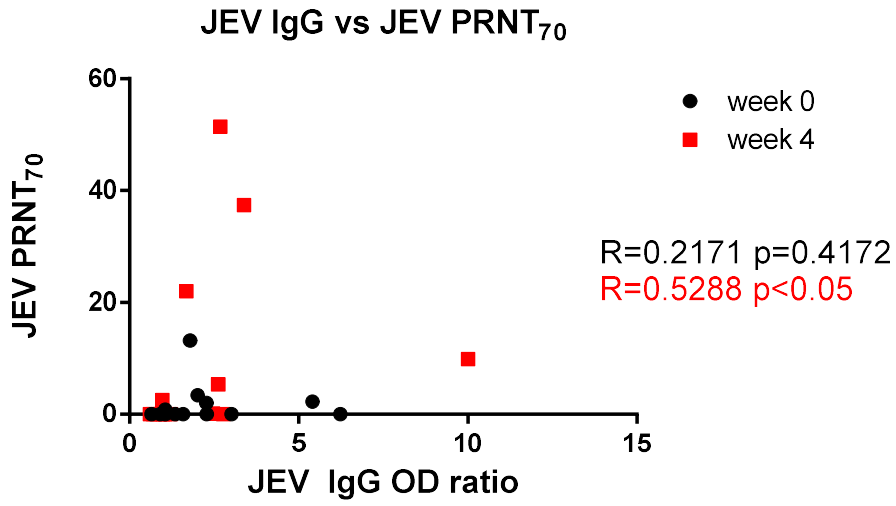


Figure 27. Correlation among JEV IgG and $PRNT_{70}$

Correlation among $PRNT_{70}$ and OD ratio of IgG specific to JEV at week 0 and 4 (Spearman test).

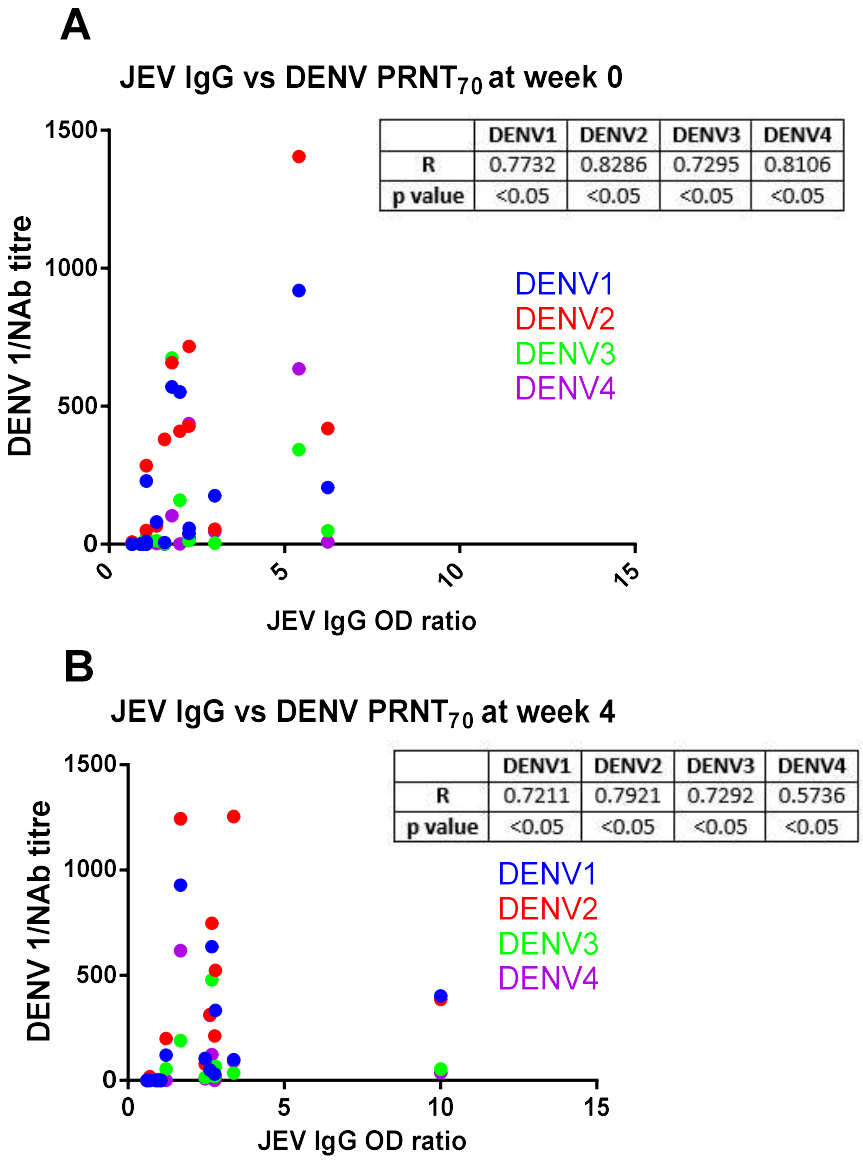


Figure 28. Correlation among JEV IgG and DENV PRNT$_{70}$.

Correlation among DENV1-4 PRNT$_{70}$ and OD ratio of JEV IgG at week 0 (A) and 4 (B) (Spearman test).

DENV IgG was detected in 10 participants (62.5%) at baseline. All of them also had DENV NAb detected at least against one serotype. Only 2 participants had DENV NAb detected but they were DENV IgG negative (018c1k1 was DENV 2 positive by NAb at both time point, week 0 and week 4, and 022c3k1 was DENV1 positive at baseline). DENV IgG values remained positive 4 weeks after vaccination without

significant variation of the OD ratio. Moreover, samples collected at week 4 for the subjects who were DENV IgG negative at baseline remained negative (table 29).

Table 29. OD ratio IgG DENV specific measured at baseline and 4 weeks after vaccination.

DENV IgG ELISA					
Volunteer ID	**Week 0**		**Week 4**		**Vaccine responder by $PRNT_{70}$**
	OD ratio	**ELISA readout**	**OD ratio**	**ELISA readout**	
005c3k1	4.71233	Positive	4.53425	Positive	Yes
001c1k1	4.33521	Positive	4.34811	Positive	Yes
023c1k1	4.80902	Positive	4.50443	Positive	Yes
015c3k1	4.40935	Positive	4.28606	Positive	No
023c3k1	0.02337	Negative	0.02579	Negative	No
020c1k1	2.871072	Positive	2.99194	Positive	No
008c3k1	2.93392	Positive	3.02176	Positive	No
019c3k1	1.70105	Positive	2.59226	Positive	No
007c1k1	4.15471	Positive	4.1805	Positive	No
017c1k1	0.07977	Negative	0.06285	Negative	No
022c3k1	0.06044	Negative	0.04432	Negative	No
018c1k1	0.11039	Negative	0.07897	Negative	No
012c3k1	3.99436	Positive	3.96696	Positive	No
020c3k1	0.02256	Negative	0.0145	Negative	No
004c1k1	0.070105	Negative	0.11442	Negative	No
010c3k1	3.66156	Positive	NA	NA	No

Overall, a strong and significant correlation among $PRNT_{70}$ values (the highest titre among the 4 DENV serotypes was used for this analysis) and DENV IgG OD ratio at week 0 ($r=0.8609$, $p<0.05$) and 4 ($r=0.8793$, $p<0.05$) was observed (figure 29A) (Spearman test). When correlating DENV IgG with DENV $PRNT_{70}$ for each DENV serotype, although good correlation was observed for all DENV serotypes, a stronger correlation was observed against DENV1 and DENV2 which are the serotypes with

the highest NAb titres (figure 29 B and C). As shown above, and in previous work, pre-existing flavivirus antibody exposure may increase the immunogenicity of a secondary flavivirus vaccine[243]. To confirm the observations described above, DENV IgG levels were compared between those who mounted NAb responses to LAJV (defined by $PRNT_{70}$) and those who did not. Those participants who responded to JE vaccination had significantly higher OD ratios for DENV IgG at baseline (figure 30) (p value <0.05, Mann Whitney U test). To confirm that this difference was not due to the fact that no DENV seronegative participants made NAb responses to LAJV, a similar analysis was conducted excluding all the participants who were DENV seronegative. Interestingly, a significant difference could still be observed with higher DENV IgG OD ratios at baseline in those who responded (figure 30) (p value <0.05 by Mann Whitney U test). These data are in agreement with the NAb data and indicate that a higher titre of DENV IgG may help the antibody response to LAJV. In addition, considering the cross-reactivity detected by the JEV IgG ELISA, the higher OD ratio of DENV IgG in those who responded is consistent with the hypothesis that higher OD ratio of JEV IgG at baseline is simply a reflection of the cross-reactivity of the assay with DENV. If this were the case DENV and JEV IgG should be strongly correlated. Interestingly, testing this showed a very strong correlation between JEV and DENV IgG OD ratio at both week 0 (Spearman test, $r=0.91$, $p<0.001$) and 4 (Spearman test, $r=0.80$, $p<0.001$) (figure 31) further confirming cross-reactivity with DENV detected by the JEV IgG assay.

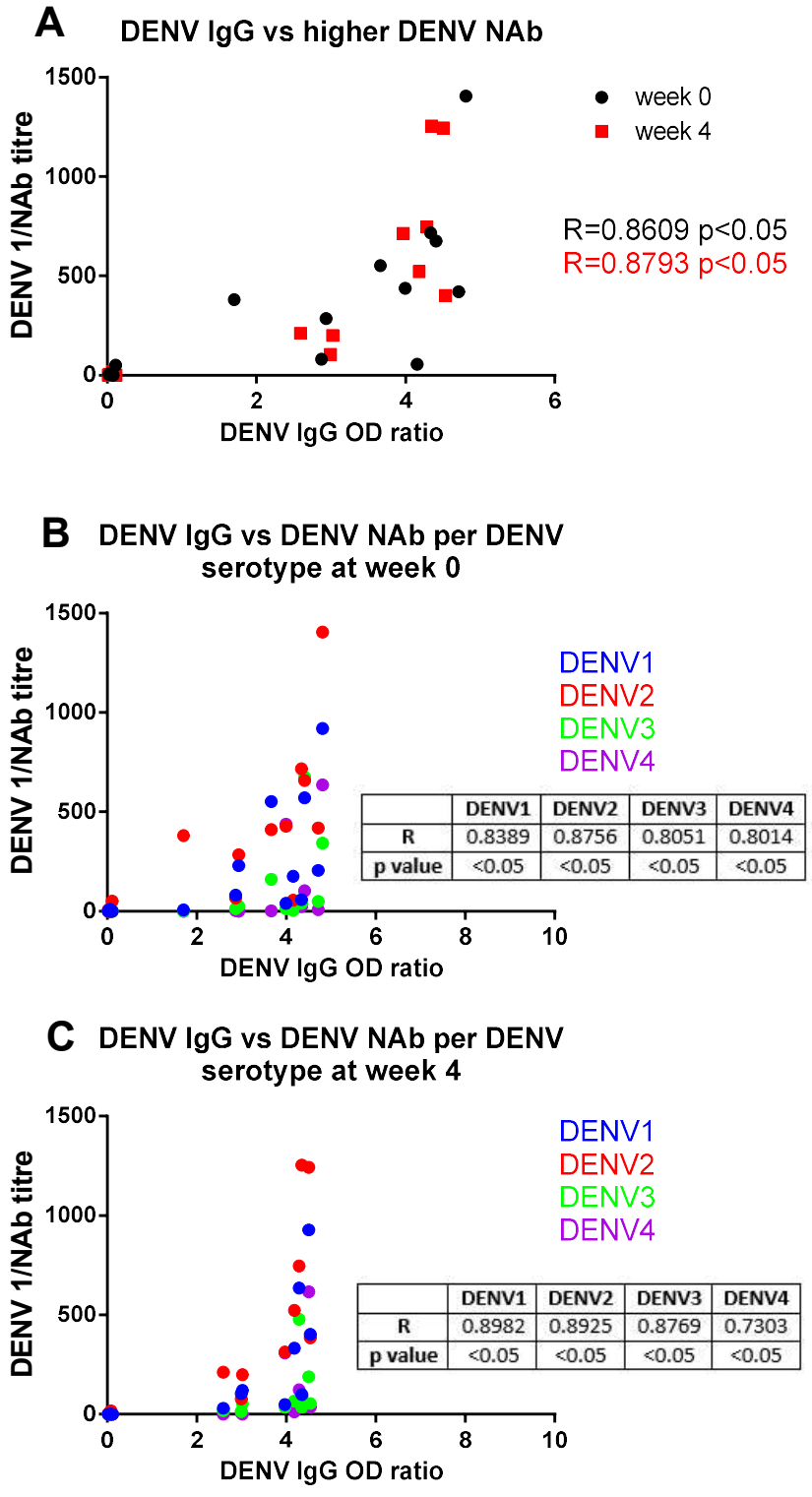


Figure 29. Correlation between DENV IgG and PRNT$_{70}$

Correlation among DENV IgG OD ratio and PRNT$_{70}$ at week 0 and 4 using the higher DENV NAb (A) or for each DENV serotype (B and C for week 0 and 4 respectively) (Spearman test).

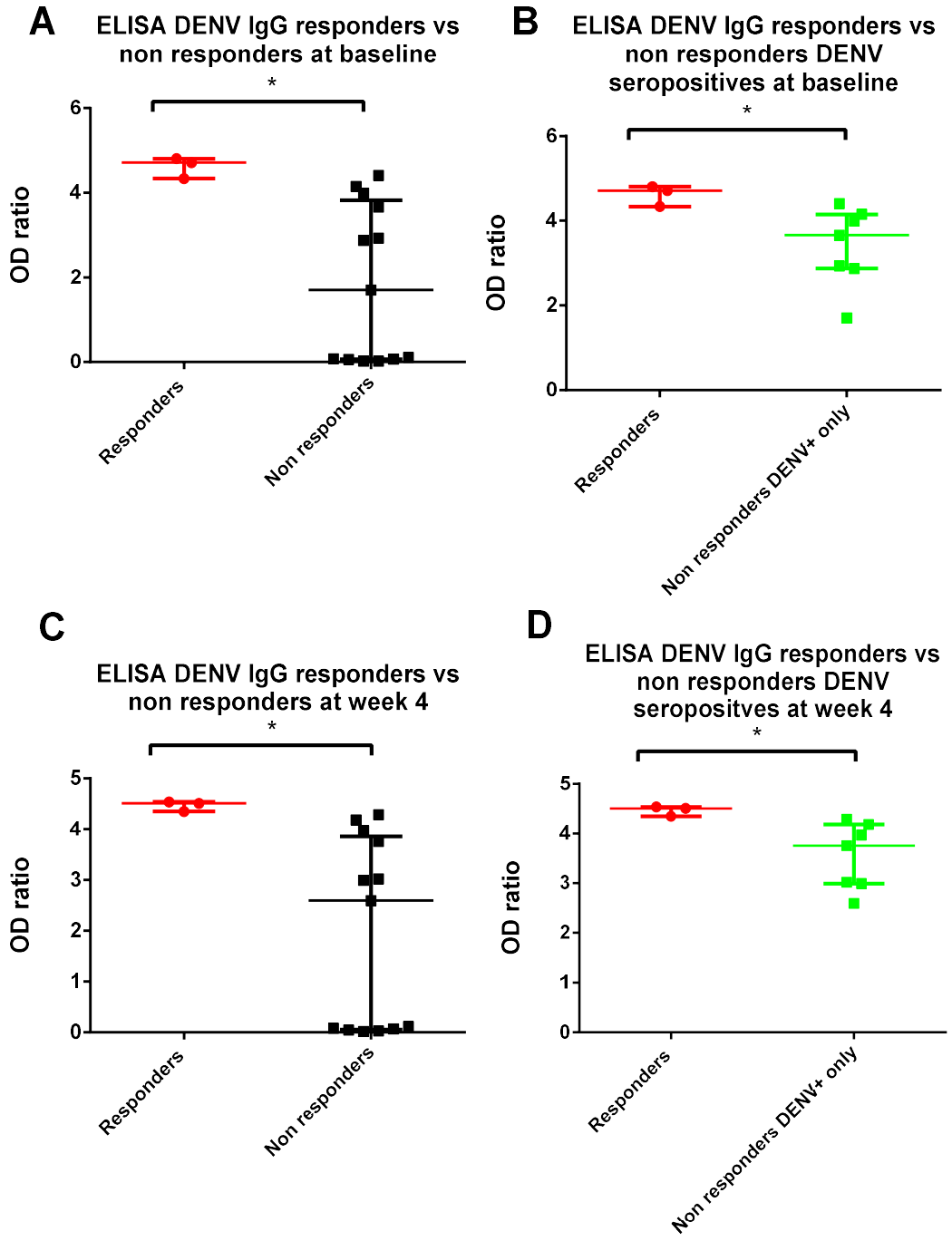


Figure 30. DENV IgG and response to the LAJV (by NAb).

OD ratio of IgG specific to DENV grouped according to the responsiveness to JEV vaccination (by $PRNT_{70}$) (A and C for week 0 and 4 respectively) and excluding the DENV seronegative participants (B and D for week 0 and 4 respectively). Asterisks indicate p value <0.05 analysed by Mann-Whitney test. Error bars represent median and interquartile range.

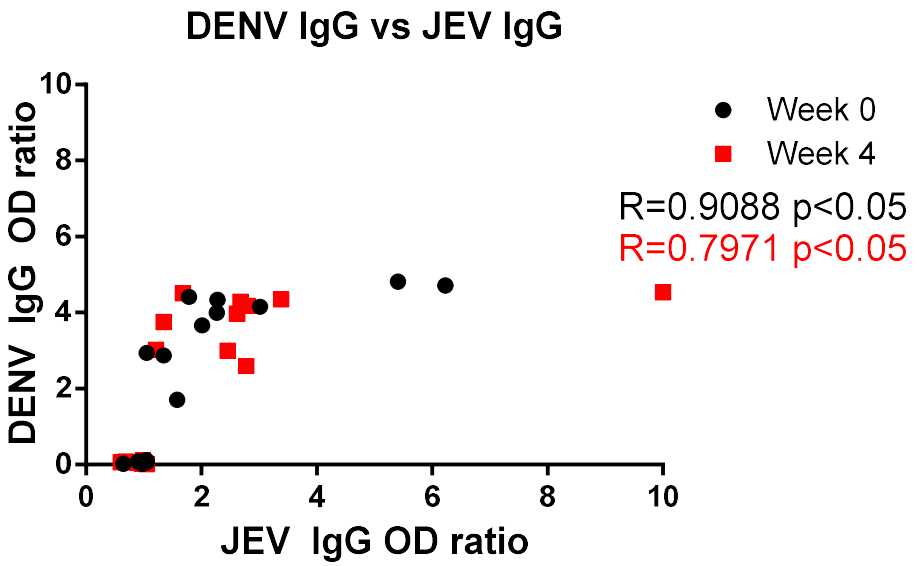


Figure 31. Correlation among DENV AND JEV IgG.

Correlation between OD ratio of DENV IgG and JEV IgG at both week 0 and 4 (Spearman test).

Individual antibody responses to LAJV – evidence of original antigenic sin

The small size of this study limited the ability to draw conclusions as to the reasons why immunogenicity (at least in terms of NAb response) was poor in this population. However, one hypothesis we wished to investigate was whether DENV exposure interfered with the response to LAJV through OAS, indicated by a participant with pre-existing DENV exposure mounting higher a DENV NAb than JEV NAb response to LAJV. As described above, DENV exposure appeared to have the opposite effect, that is DENV2 NAb levels at baseline were markedly higher in responders, achieving statistical significance even with this small sample size. There was no evidence of OAS in the cohort overall, although DENV4 NAb did show a trend to rising after LAJV, but during the course of the work it became apparent that there was marked variation in the responses of different participants. Although the small numbers impeded the ability to find any association of OAS with vaccine non-response, if observed, we could conclude that an OAS interaction exists between DENV and JEV in this cohort.

In order to test the hypothesis that OAS occurs after LAJV, responses were analysed for each participant individually. OAS was defined as a greater increase in NAb titre

against any DENV serotype than against JEV following LAJV. In addition, at least a 4-fold increase in DENV NAb titre should be observed following vaccination.

Among those who made NAb responses, 001c1k1 showed greatest fold change of $PRNT_{70}$ JEV titre (7.48 fold increase). This participant was seronegative at baseline for JEV and the NAb response peaked at week 4 with a $PRNT_{70}$ of 37 (figure 32A). However, at week 8, JEV $PRNT_{70}$ dropped below 10. JEV ELISA IgM and IgG OD ratios also showed a small increase (1.5 fold, figure 32B). Additionally, at week 0, this participant was seropositive for all DENV serotypes with the highest NAb titre obtained against DENV2 (717) followed by the other serotypes which had similar NAb titre (58, 39 and 23 for DENV1, DENV3 and DENV4 respectively). An increase in $PRNT_{70}$ against all DENV serotypes was observed following vaccination. In particular, a rise in NAb titre was detected at week 4 for DENV1, DENV2 and DENV4 and for all DENV serotypes at week 8. The increase in NAb titre against DENV4 with a 3.9 fold change at week 4 and 2.9 at week 8.

In addition, to further investigate the antibody response, plasmablasts (newly responding B cells) were measured in blood after vaccination. This cell population has been described to increase in blood after vaccination[266]. In this volunteer, a 1.8 fold increase in the plasmablast population measured by flow cytometry was observed at week 2 for this participant (figure 32C).

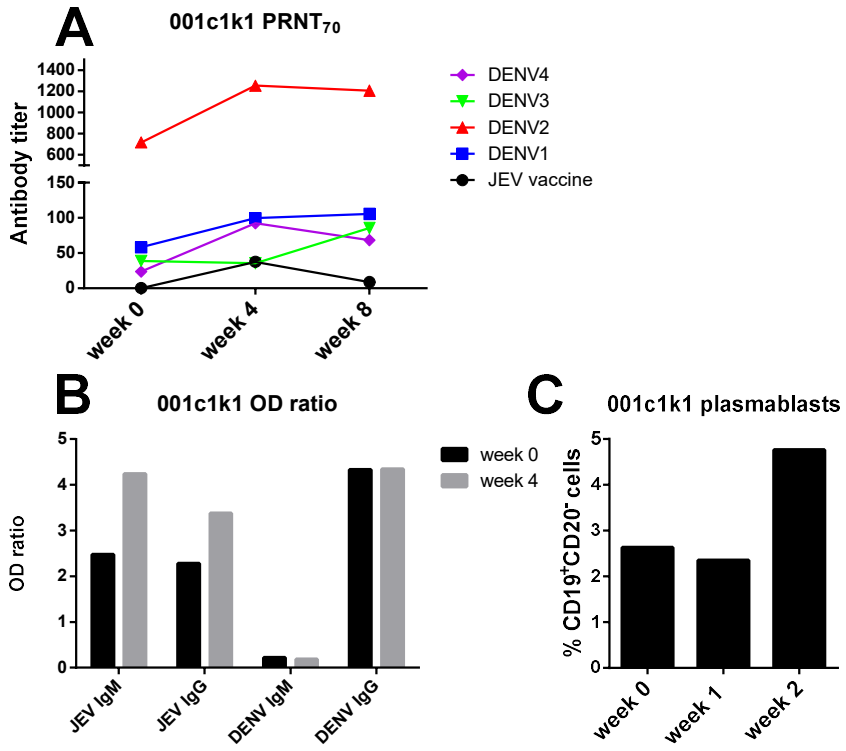


Figure 32. Summary of antibody response to LAJV in 001c1k1.

Summary of antibody response to LAJV in 001c1k1 by $PRNT_{70}$ (JEV and DENV1-4 at week 0, 4 and 8, reciprocal titre) (A), IgG and IgM ELISA (JEV and DENV at week 0 and 4, OD ratio) (B) and plasmablast response (at week 0, 1 and 2, percentage of $CD19^{+}CD20^{-}CD38^{high}CD27^{high}$ cells) (C).

The second highest NAb response to the vaccine was observed in 023c1k1 with a 4.4 fold increase at week 4 (figure 33A). JEV ELISA IgM did not change between week 0 and week 4, whereas IgG at week 0 which was strongly positive became negative at week 4 (figure 33B). Regarding DENV specific NAb, 023c1k1 was positive at baseline against all serotypes with reciprocal $PRNT_{70}$ of 919, 1405, 343 and 636 for DENV1, DENV2, DENV3 and DENV4 respectively. DENV specific IgG ELISA was also positive at baseline. No relevant increase of DENV NAb titre was observed for this participant following vaccination. Plasmablasts staining showed an expansion of 2.1 fold following vaccination (figure 33C).

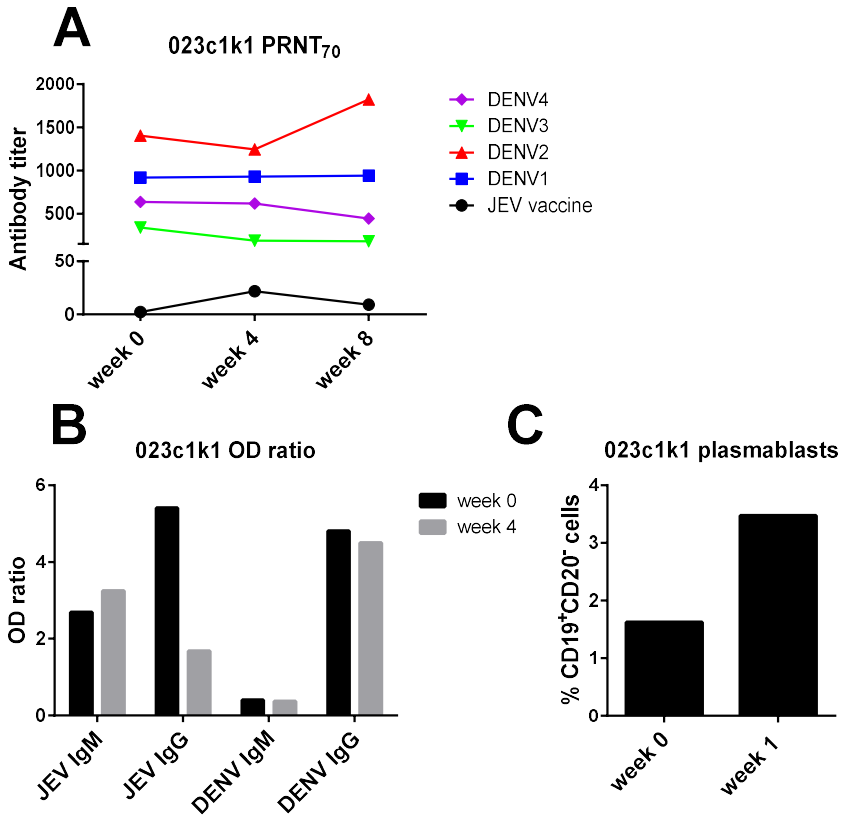


Figure 33. Summary of antibody response to LAJV in 023c1k1.

Summary of antibody response to LAJV in 023c1k1 by $PRNT_{70}$ (JEV and DENV1-4 at week 0, 4 and 8, reciprocal titre) (A), IgG and IgM ELISA (JEV and DENV at week 0 and 4, OD ratio) (B) and plasmablast response (at week 0 and 1, percentage of $CD19^{+}CD20^{-}CD38^{high}CD27^{high}$ cells) (C).

The final participant who seroconverted following vaccination was 005c3k1. Despite a strongly positive JEV IgG ELISA before vaccination, no JEV neutralisation was observed at baseline by $PRNT_{70}$. Four weeks after vaccination, JEV NAb titre rose to 10 and peaked at 8 weeks after vaccination with a $PRNT_{70}$ value of 12 (figure 34A). A rise of IgM and IgG was observed by JEV ELISA with a 1.9 and 1.6 fold rise respectively of the OD ratio between week 0 and week 4. This participant was DENV seropositive at baseline, with NAb detected against DENV1, 2 and 3. Interestingly, following JEV vaccination, a rise in NAb titre against DENV1 and DENV4 was observed, though the DENV IgG ELISA titre did not change. DENV1 NAb titre rose 1.9 fold at week 4 and 1.5 fold at week 8 (figure 34B). The fold change increase was

higher for DENV4 with an increase of 4 and 6 fold measured at week 4 and 8 respectively. The higher response observed against DENV4 than to JEV (which was the antigenic stimulus) demonstrates OAS in this participant. No concurrent DENV infection was observed during the period of observation as confirmed by the negative results of ELISA IgM DENV specific at week 0 and 4. Insufficient samples were available from this participant for plasmablast measurement.

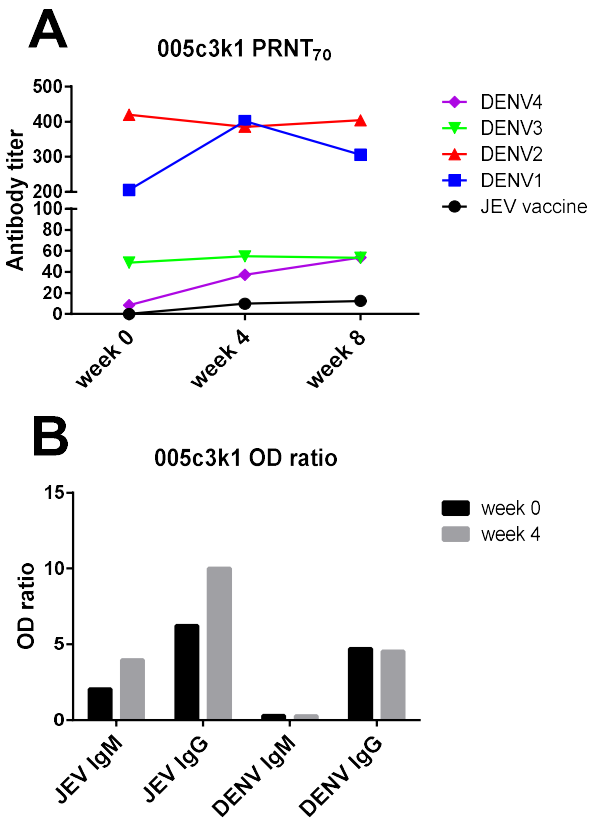


Figure 34. Summary of antibody response to LAJV in 005c3k1.

Summary of antibody response to LAJV in 005c3k1 by $PRNT_{70}$ (JEV and DENV1-4 at week 0, 4 and 8) (A), IgG and IgM ELISA (JEV and DENV at week 0 and 4) (B).

A further 9 volunteers had DENV NAb at baseline, but did not mount NAb response to the vaccine. Of these, two volunteers, 007c1k1 and 019c3k1 showed increase in DENV NAb following LAJV.

Only the time points week 0 and week 4 were available for 007c1k1. Interestingly, this participant tested JEV IgM positive at baseline though only a very modest change was

seen (1.4 fold increase) following vaccination. JEV specific IgG was equivocal at both time points and did not change after vaccination. DENV specific IgG and $PRNT_{70}$ at week 0 indicated this subject was DENV seropositive at baseline, with NAb against DENV1, 2 and 4 (figure 35 A). A large increase in DENV2 NAb titre was observed following JE vaccination, DENV1 NAb also increased and there was seroconversion to DENV3, again indicating OAS (figure 35A). It is interesting to note also the magnitude of the response with a 1.89, 9.4 and 13.3 fold increase of the antibody titre respectively for DENV1, DENV2 and DENV3. The response following the vaccination was accompanied by plasmablast expansion that peaked at week 1 with a 3.6 fold increase (figure 35C).

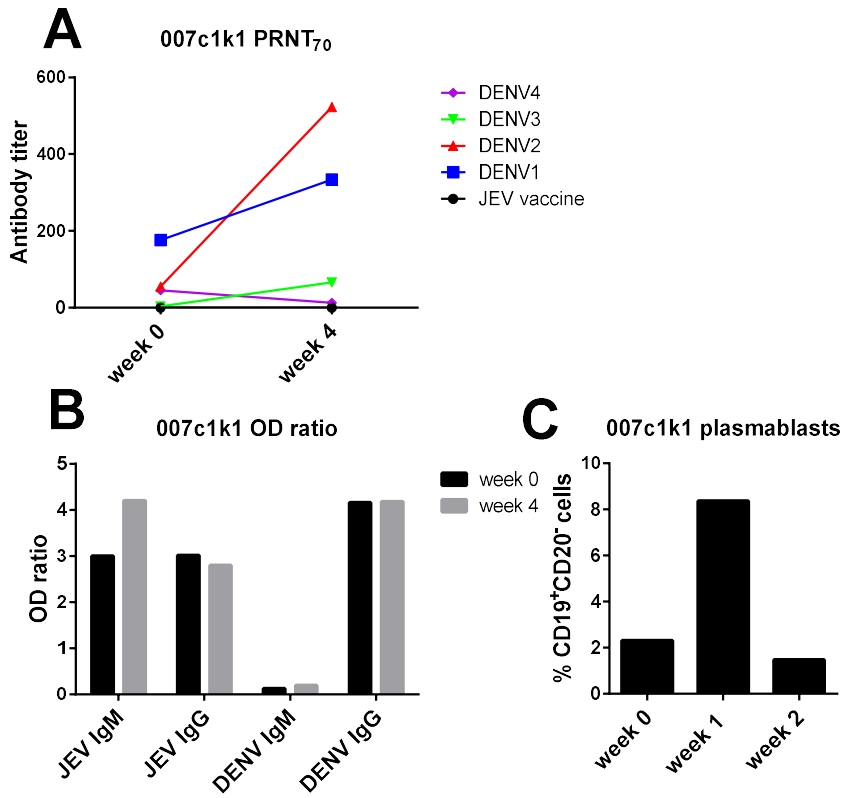


Figure 35. Summary of antibody response to LAJV in 007c1k1.

Summary of antibody response to LAJV in 007c1k1 by $PRNT_{70}$ (JEV and DENV1-4 at week 0 and 4) (A), IgG and IgM ELISA (JEV and DENV at week 0 and 4) (B) and plasmablast response (at week 0, 1 and 2, percentage of $CD19^{+}CD20^{-}CD38^{high}CD27^{high}$ cells) (C).

For participant 019c3k1, there was no change in JEV IgM following vaccination, though there was a small rise (1.7 fold) in JEV IgG ELISA OD ratio at week 4. DENV specific NAb at baseline was observed only against serotype 2. Interestingly, this participant seroconverted for DENV1 and 3. $PRNT_{70}$ values for DENV 1 and 3 were <10 before JE vaccination; 4 weeks after vaccination reciprocal NAb titres for DENV 1 and 3 were 31 and 20 respectively, indicating OAS, or at least a cross-reactive response which neutralised some DENV serotypes better than JEV. Plasmablast staining showed expansion at week 1 showed a 3.8 fold increase over baseline (figure 36).

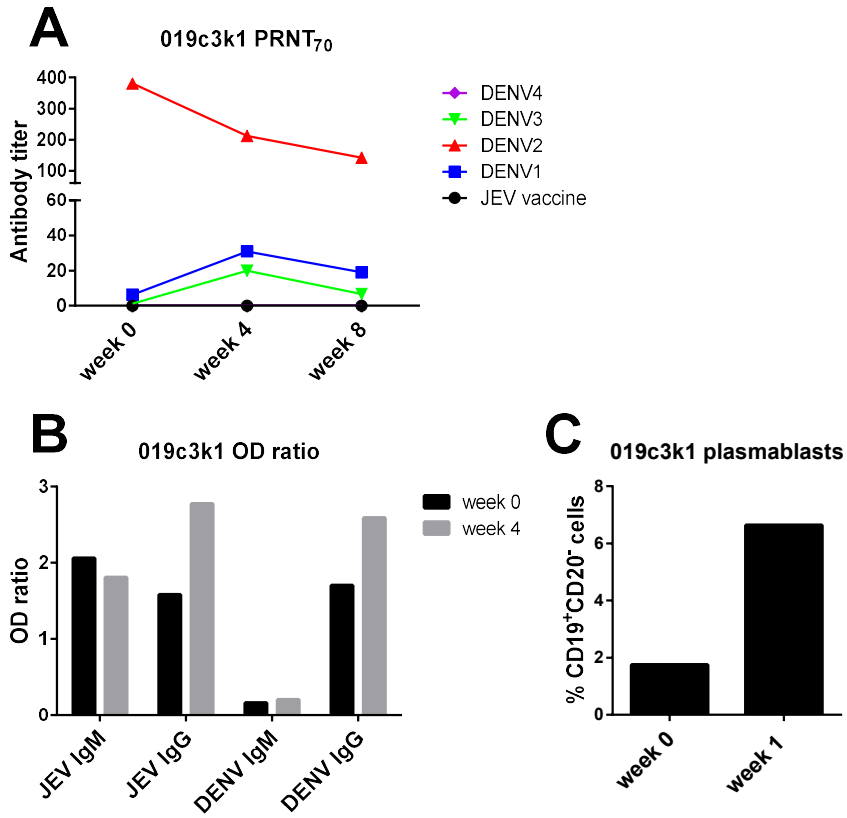


Figure 36. Summary of antibody response to LAJV in 019c3k1.

Summary of antibody response to LAJV in 019c3k1 by $PRNT_{70}$ (JEV and DENV1-4 at week 0, 4 and 8) (A), IgG and IgM ELISA (JEV and DENV at week 0 and 4) (B) and plasmablast response (at week 0 and 1, percentage of $CD19^{+}CD20^{-}CD38^{high}CD27^{high}$ cells) (C).

The remaining 7 participants who had DENV NAb at baseline, did not respond to LAJV with detectable NAb by $PRNT_{70}$ and did not show any significant change in DENV NAb titres after vaccination. Of these participants, 012c3k1 showed a >2 fold increase in the plasmablast population at 14 days post LAJV (figure 37).

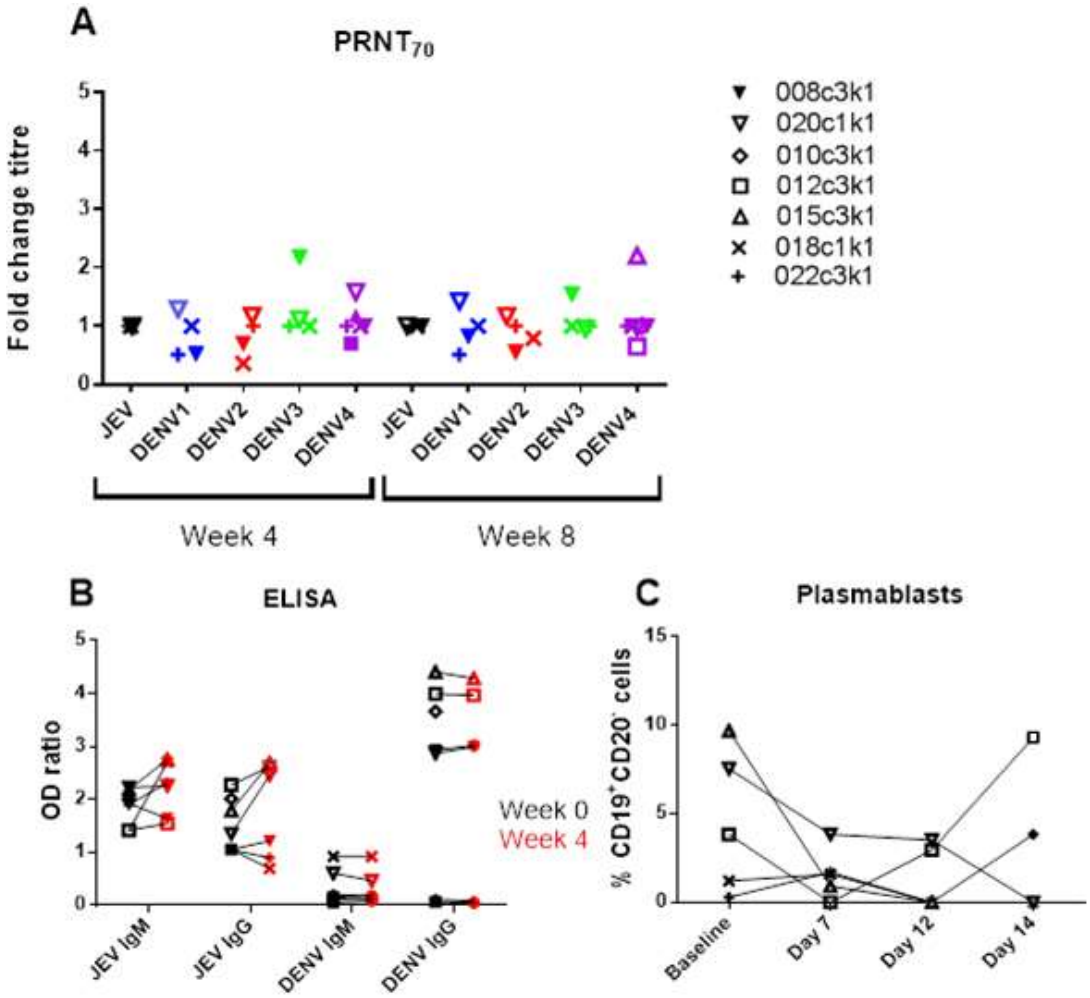


Figure 37. Summary of antibody response to LAJV in 008c3k1, 012c3k1, 015c3k1, 010c3k1, 022c3k1, 020c1k1 and 018c1k1.

Summary of antibody response to LAJV in 008c3k1, 012c3k1, 015c3k1, 010c3k1, 022c3k1, 020c1k1 and 018c1k1 by $PRNT_{70}$ (fold change of NAb titre for JEV and DENV1-4 at week 0, 4 and 8) (A), IgG and IgM ELISA (JEV and DENV at week 0 and 4) (B) and plasmablast response (at baseline and day 7, 12 and 14, percentage of $CD19^{+}CD20^{-}CD38^{high}CD27^{high}$ cells) (C).

Finally, 4 participants had no DENV antibodies at baseline and did not make detectable NAb response to LAJV measured by $PRNT_{70}$. However, three of these participants

had IgM ELISA OD ratios that at least doubled 4 weeks after vaccination, one of whom also showed an increase in the plasmablast population after vaccination (figure 38).

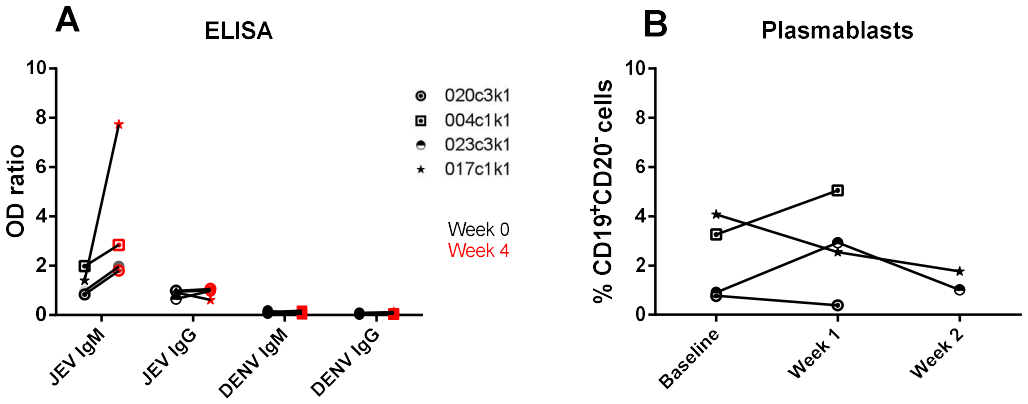


Figure 38. Summary of antibody response to LAJV in 023c3k1, 020c3k1, 004c1k1 and 017c1k1.

Summary of antibody response to LAJV in 023c3k1, 020c3k1, 004c1k1 and 017c1k1 by IgG and IgM ELISA (JEV and DENV at week 0 and 4) (A) and plasmablast response (at week 0, 1 and 2, percentage of $CD19^{+}CD20^{-}CD38^{high}CD27^{high}$ cells) (B).

Overall, OAS was observed in 3 participants and for all DENV serotypes. In particular, OAS was detected in 2 participants for DENV3, in 1 participant for DENV1, DENV 2 and DENV4. Interestingly, OAS was never observed for the DENV serotype with the highest NAb titre for each participant.

Although only 3 participants developed OAS, OAS occurred among participants who developed NAb and who did not (figure 39 and table 30). Approximately 30% of the participants who were DENV seropositive (by IgG or PRNTs) showed OAS (table 31 and 32). As the study was small and only three participants responded to the JE vaccine (by NAb), analysis of the difference of the JEV neutralising GMT among responders who developed OAS versus those who did not was not possible. However, it is possible to conclude that DENV NAb influences the response to LAJV, and that LAJV influences DENV NAb levels.

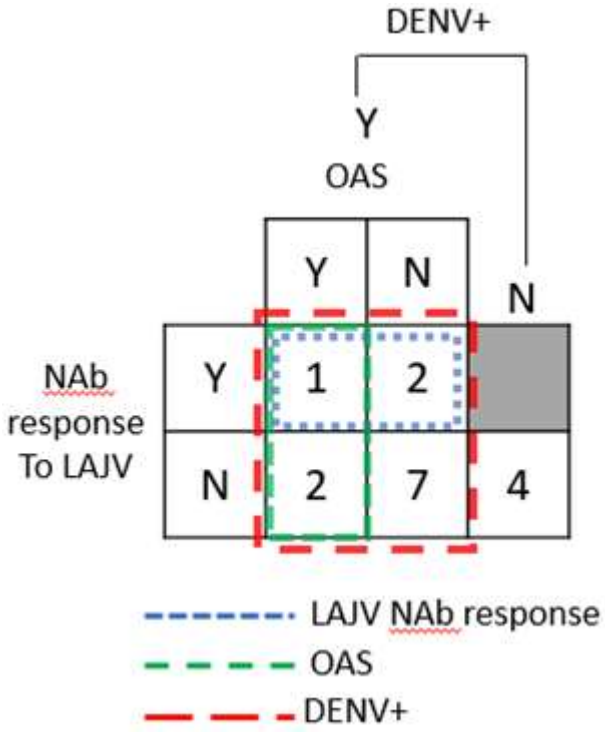


Figure 39. Schematic representation of the overall NAb response and OAS observed in this study.

Table 30. Prevalence of participants who developed OAS among responders and non-responders (by NAb) to LAJV administration

	Sin	**Non sin**	**Total**
Responder	1 (33.3%)	2 (66.6%)	3 (100%)
Non responder	2 (15.4)	11 (84.6%)	13 (100%)
Total	3	13	16

Table 31. Prevalence of participants who developed OAS among DENV seropositive and DENV seronegative participants according to IgG DENV ELISA.

	Sin	**Non sin**	**Total**
DENV seropositive (by IgG)	3 (30%)	7 (70%)	10 (100%)
DENV seronegative (by IgG)	0 (0%)	6 (100%)	6 (100%)
Total	3	13	16

Table 32. Prevalence of participants who developed OAS among DENV seropositive and DENV seronegative participants according to $PRNT_{70}$.

	Sin	**Non sin**	**Total**
DENV seropositive (by $PRNT_{70}$)	3 (25%)	9 (75%)	11 (100%)
DENV seronegative (by $PRNT_{70}$)	0 (0%)	4 (100%)	5 (100%)
Total	3	13	16

Overall, we are not able to present any conclusive evidence that OAS adversely affects JE vaccination. However, OAS does exist after LAJV, a finding not previously described.

Discussion

Since the LAJV has been introduced, it has been administrated to more than 700 million children[267]. The vaccine has been extensively shown to be safe and well tolerated by children as young as 8 months as well as by older people 15 years of age[267]. The safety of LAJV among the 17 participants vaccinated was previously reported; 6 adverse events were observed in 3 subjects and included fever, headache, myalgia, sore throat, dry cough and dizziness[158].

In JE endemic countries a single dose of LAJV SA14-14-2 has high efficacy[99,268]. However, one case control study conducted in China found 80% and 97.5% efficacy of 1 and 2 doses, respectively[101]. Seroconversion following JE vaccination is usually analysed by $PRNT_{50}$, a value equal or above 10 is considered positive, and taken to indicate protection. Several studies have looked at seroconversion following LAJV, and have showed a range of seroconversion rates from 80-99% [97, 98]. In this study a lower seroconversion rate was found. As $PRNT_{70}$ values were used, this may have decreased the number of volunteers who seroconverted. In our previous published work on this cohort of participants, a seroconversion rate of 62.5% was measured by $PRNT_{50}$[158]. Another reason for the low seroconversion rate was the population to whom LAJV was administered in this study, which consisted of Indian adults with a median age of 25 years (range 20-39), whereas the vaccine is usually used in children. Interestingly, a recent study also conducted in India also found a seroconversion rate

of 57.69 and 39.74% respectively at week 4 and 8 in adults following JEV vaccination with SA14-14-2[81,102], a very similar rate (62.5%) to that observed in this population using the equivalent measure ($PRNT_{50}$)[158]. Another study, conducted in Indian adults, described an 85.5% seroconversion rate in subjects that were JEV seronegative at baseline[269]. Interestingly, however, a vaccine efficacy of only 43.8% was observed in a case control study conducted in Indian children[103].

This study found a low GMT among responders at 4 and 8 weeks after vaccination. In a similar age group to this study, Singh *et al.* also reported low GMT against JEV in Indian volunteers vaccinated with the LAJV (36.50 at week 4 and 31.84 at week 8 calculated by $PRNT_{50}$). The antibody response in this study peaked at week 4 and declined at week 8, similar to the findings of two other studies [81, 269].

Dengue cases have gradually increased over recent years in India[270]. The majority of the participants in this study were DENV exposed, as shown by IgG and NAb specific to DENV. The higher GMT and prevalence at baseline of antibodies to DENV2 is consistent with previous epidemiological data from Karnataka state[265] that indicate greater circulation of DENV2 than other serotypes.

Secondary infection with any DENV serotype is an important risk factor for developing a more severe form of dengue disease. For this reason, many studies have focused on understanding the antibody response during a secondary DENV infection. Antibody responses to other flaviviruses during a secondary flavivirus infection have been less well studied. Previous work on DENV can be partially translated in understanding these data as protein identity among DENV serotypes is around 70% whereas protein identity between JEV and any DENV serotype is around 50%. A secondary heterologous DENV infection is dominated by a cross-reactive antibody response[173, 271]. The major target of these antibodies is the virion where they bind mainly to prM and E proteins. Anti-prM antibodies were shown to be poorly neutralising[181]. Anti-E antibodies have different degrees of neutralisation and cross-reactivity, depending upon their epitope specificity. The most well characterized, poorly neutralising, highly cross-reactive antibodies were demonstrated to bind the FL region of the E protein: a highly conserved region among all flaviviruses[261, 182]. Antibodies that bind the E protein FL in secondary DENV infection have some neutralising activity against DENV[183]. These antibodies bind the FL of ZIKV with

high affinity, but they do not neutralise at all[262]. It is possible that a similar class of cross-reactive, but non-neutralising, antibodies were made after LAJV in these volunteers, which could neutralise DENV somewhat, but did not neutralise JEV.

Both the antibody response and the T cell response during secondary heterologous DENV infection can exhibit OAS[195 202 264]. In this study, OAS was observed in the NAb response of 3 participants following JE vaccination.

This is the first study demonstrating this phenomenon with JEV and DENV, viruses more distantly related than DENV serotypes.

The mechanism at the base of the phenomenon of OAS is still unknown. One hypothesis suggests that there is competition for B cell receptor antigen binding among naïve and memory B cells with the latter outcompeting naïve cells by being present in higher number and having a lower activation threshold[193]. Another hypothesis invokes activation of regulatory T (Treg) cells, primed by the original and able to recognise the second antigen, which reduce the amount of antigen loaded on antigen presenting cells and limit the activation of naïve B cells[198]. Neutralisation of the anti-inflammatory cytokine IL-10 prevented OAS in an animal model, although Treg cells were not found to be the source of IL-10[200]. Previous work, which identified two strategies to block OAS, supports both hypotheses: repeated stimulation with the secondary antigen or the use of adjuvants to stimulate antigen presenting cells[199].

As the mechanism behind OAS is still unknown, it is difficult to explain why this phenomenon was observed only in some volunteers. It can be speculated that increase of the phylogenetic distance among viruses may reduce the occurrence of the phenomenon, which may depend on the original infecting strain of DENV.

OAS was first observed in influenza[191], where some animal model data suggest a biologically relevant effect on protection. In the work of Kim *et al.*, OAS was observed in mice vaccinated with different types of influenza vaccines (inactivated, deoxyribonucleic acid (DNA) and live attenuated vaccines) and it was responsible for reduced efficacy of the vaccine[193]. In humans, however, the clinical relevance of OAS is still a matter of debate. Some studies found no interference by OAS in vaccine efficacy or did not observe the phenomenon at all[272,194]. Decreased vaccine seroconversion due to OAS is well described[201, 273], but whether this results in lack of protection against disease is not known.

The data presented here show that although OAS occurs after JE vaccination, it did not necessarily hinder the development of anti-JEV NAb. Whether OAS can account for the poor immunogenicity of JE vaccine seen in this study, and in other studies in India, remains unclear. We only studied a small number of subjects, and a larger study may yet reveal a role for OAS in hindering the NAb response to JE vaccine.

More participants responded to the vaccine by measuring IgM specific to JEV although only a modest increase was observed following LAJV. This is in agreement of the work of Sohn *et al.* where only 13% of the children immunized with 1 dose of the LAJV produced IgM specific to JEV[274]. The results presented in this thesis also suggested a non-neutralising response mounted by the participants with a serological profile of JEV IgM+ JEV PRNT-. Whilst this profile could be explained by an increase in cross-reactive non-neutralising antibodies, such as FL antibodies, this response was also observed in DENV seronegative participants. Our data regarding JEV specific IgG are difficult to interpret. Higher JEV IgG levels were observed in the participants who were DENV seropositive. Additionally, one participant who was JEV IgG positive became negative following vaccination, questioning the reliability of the assay. Furthermore, it is important to note that the assay used was developed to detect natural exposure and not vaccine response, as stated by the manufacturer. The most likely confounding factor is the cross-reactivity of this assay with DENV antibodies. Overall, these data confirmed how difficult it can be to discriminate between anti-JEV or anti-DENV antibodies, or more generally among flaviviruses, in countries where different flaviviruses co-circulate.

Measurement of IgG and IgM against DENV by ELISA did not show any increase following vaccination despite observing an increase of DENV NAb in some participants. This indicated that the ELISAs detected antibodies with different specificity than those measured by $PRNT_{70}$. An explanation for these data could be the use of diagnostic ELISA tests (the antigen used is not mentioned) that have probably been designed to avoid cross-reactive antibodies, or could employ the secreted NS1 protein as antigen.

Another important finding of this study was the higher titre of IgG and NAb against DENV in those participants who responded to LAJV. These data are in agreement of the work of Chan *et al.* where cross-reactive and enhancing antibodies derived by JE

vaccination (inactivated vaccine) increased the immunogenicity of yellow fever vaccine by increasing infectivity, viraemia and the pro-inflammatory response[243]. The role of pre-existing antibodies in flavivirus infection has been investigated in different studies. Interesting data came from the first human clinical trial of a dengue vaccine where a chimeric DENV was administrated to volunteers in endemic countries (prM and E of each dengue serotype with a backbone of the live attenuated yellow fever vaccine)[234–236]. The vaccine, which did not show high efficacy, enhanced DENV infection in volunteers who were DENV seronegative at the time of vaccination[238,275]. Several studies have looked at the role of these cross-reactive antibodies among flaviviruses of different serogroups in natural infection. Anderson *et al.* associated pre-existing NAb to JEV with a higher rate of symptomatic dengue infection upon subsequent exposure[248]. This was not the case for those who had received inactivated JE vaccine, suggesting that natural exposure accounted for the phenomenon. More recently, many studies have described the interaction of DENV antibodies with ZIKV. Although there is strong evidence that cross-reactive antibodies, including anti-FL antibodies, promote enhancement of ZIKV *in vitro*[262], their role *in vivo* is still unclear. Studies using non-human primate and human studies have shown no evidence of ADE *in vivo*[276,277]. Consistent with this are observations that previous DENV is protective, and not detrimental, for subsequent ZIKV infection[278,279].

In this project the only protective mechanism studied was direct neutralisation of the virus by the Ab response. Direct neutralisation is believed to be the main mechanism of protection by antibodies in flavivirus infection, although a role for other mechanisms is not excluded. Other antibody mediated anti-viral mechanisms are antibody-dependent cellular cytotoxicity (ADCC), complement fixation, and opsonisation. Although a previous study demonstrated that ADCC kills DENV infected cells *in vitro*[280], García *et al.* found ADCC occurring only with serum samples collected from severe cases of dengue[281]. More work is still required to elucidate the implications of this phenomenon in the protection from the disease. No work has been published to date on ADCC in JE, therefore more experiments looking at this aspect of the antibody response are necessary.

The role of the complement fixation by anti-flavivirus antibodies has been studied mainly in WNV and DENV with both protective and pathogenic effects described. *In vitro*, WNV replication is increased through macrophage complement receptor 3 in the

presence of WNV specific IgM and complement[282]. On the contrary, Melhlop *et al.* showed that the complement component C1q in presence of IgG subclasses 1 and 3 could abolish ADE *in vitro* and *in vivo*[283].

The main limitation of this study is the sample size which limits the conclusions that can be drawn. In addition, the ELISAs performed in this study were not quantitative in nature but qualitative and are intended for diagnostic purposes. Although these assays gave helpful information about the serological status of the participants, including the non-neutralising component of the response, the IgM and IgG JEV kits were not designed to detect a response to JE vaccine. Moreover, the antigen used to bind the antibodies is not specified making the data difficult to interpret. ELISA using whole JEV and DENV virions as antigen may have helped to have a more complete overview of the antibody response and its degree of cross-reactivity.

Most of the participants had detectable T cell proliferation responses to JEV at baseline, whereas NAb at baseline were observed only in one participant[158]. Although DENV cross-reactive T cells may give rise to proliferation responses, T cell proliferation responses were observed also in DENV seronegative volunteers. This could represent a model of immunity with memory T cells providing protection even in the absence of NAb[158,284].

In summary, this study showed low immunogenicity of the LAJV in Indian adults. Larger studies, or studies with clinical disease as the end point, are therefore required to confirm these observations and define their clinical relevance. Additionally, this work describes the phenomenon of OAS occurring among the LAJV and DENV in some participants for the first time. However, we were unable to determine any impact of OAS on JE vaccination. Finally, this study suggests that pre-existing DENV antibodies may contribute to improved seroconversion after JE vaccine.

Chapter III: T cell response to live attenuated Japanese encephalitis vaccine

This chapter includes data obtained by other colleagues.

Figure 40 was produced by others.

I re-analysed data obtained by other colleagues to generate figures 42-46.

Figure 48, table 33 and table 36 together with my data include data previously generated by others.

Introduction

The main marker used to measure response to Japanese encephalitis (JE) vaccines is seroconversion by analysing production of neutralising antibody (NAb) against Japanese encephalitis virus (JEV). NAb play a crucial role in protection against JEV and a titre of 1:10 ($PRNT_{50}$) is considered protective. The role of T cells in JE is much less studied and is not fully understood. Similarly, a knowledge gap exists regarding the T cell response to the live attenuated JE vaccine (LAJV). Animal and human studies have implied a role for T cells in protection. Both CD4 and CD8 T cells together contributed to protection in a mouse model, although CD8 T cells were also found to be involved in pathogenesis in the central nervous system (CNS)[147 285]. Other studies have confirmed the importance of T cells in controlling JEV. Jain *et al.* demonstrated the importance of CD8 T cells in reducing mortality by adoptive transfer experiments and highlighted the importance of the lytic activity of these cells in controlling the viral infection[148]. Similar findings were observed in another study where adoptive transfer (intracerebral) of anti JEV cytotoxic T cells protected adult mice from a lethal intracerebral JEV challenge[149].

In human, Turtle *et al.* described differences of T cell responses among JEV exposed healthy individuals (asymptomatic JEV infections) and JE recovered patients. A higher frequency of CD8 responses targeting the non-structural proteins were observed in healthy JEV exposed donors in comparison to recovered JE patients where CD4 T cells responding to the structural proteins dominated the response. The quality of the CD4 T cell response was also related to the outcome of JE with polyfunctional responses being associated with complete recovery[157]. Furthermore, other studies have associated interferon-γ (IFNγ) production with good outcome in JE[156,154] again indicating that T cells have a role in protection from JE.

In previous work related to this thesis, our group described the T cell response to LAJV. Response to all JEV proteins was detected with both CD4 and CD8 T cells responding to JEV peptides. Additionally, cross-reactivity with dengue virus (DENV) and West Nile Virus (WNV) peptides was also described for some of these epitopes[158].

In another live attenuated flavivirus vaccine (yellow fever), polyfunctional T cell responses, CD4 and CD8, to all proteins were described[286 287]. The tetravalent dengue vaccine (TDV), another live attenuated vaccine developed by the National Institute of

Health, induced similar patterns of response with polyfunctional CD4 and CD8 T cells following administration[288] [289]. Interestingly, differences were found among monovalent and tetravalent vaccine administration, with T cell responses focusing on more conserved epitopes following vaccination with the tetravalent vaccine[290]. This was also observed in a mouse model transgenic for human leukocyte antigens (HLA) lacking the α/β interferon (IFN) receptors where heterologous infection with different DENV serotypes skewed the T cell response towards conserved epitopes with an impairment to develop serotype specific T cells[291].

The aims of this work were:

- To expand the work previously conducted on the same cohort of participants and to map more T cell epitopes. In so doing, to:
    1. explore whether any aspect of the T cell response to LAJV correlates with the existence of OAS in the NAb response.
    2. understand if previous T cell responses to DENV could hinder the development of new T cell responses following the administration of the LAJV.

Initial work on the analysis of the T cell response following LAJV was performed by Dr Lance Turtle and co-workers. T cell responses were measured at baseline and week 1, 2, 4, 8, 16 and 26 following vaccination by IFNγ-ELISpot. One volunteer had a detectable T cell response to NS3 (peptide ALRGLPVRY, NS3, 1739-1747) at baseline. However, proliferation responses measured using frozen cells were observed in 9 out of 13 volunteers tested in both CD4 as well as CD8 T cells before vaccination. After vaccination, T cell response was detected by IFNγ-ELISpot in 12 volunteers out of 15. Overall, the response peaked at week 2 with a geometric mean spot forming cells (SFC) per million peripheral blood mononuclear cell (PBMC) of 77.5 (figure 40) and spanned through all the JEV proteins[158].

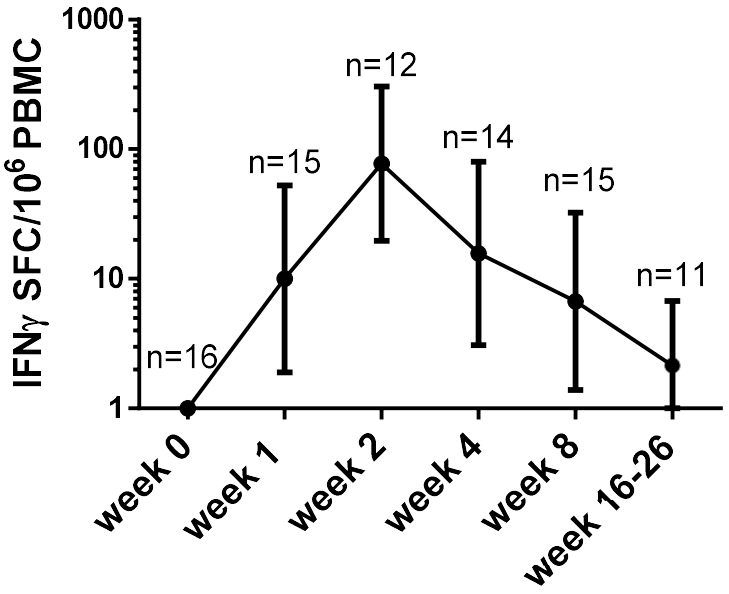


Figure 40. T cell response to JEV following LAJV.

Geometric mean of SFC per 1 million PBMC observed following LAJV with bar indicating the 95% confidence interval. PBMC were stimulated with JEV peptide pools and T cell response measured by IFNγ-ELISpot. Figure edited from Turtle et al. [158].

The work described in this chapter involved identification of T cell epitopes in PBMC collected after vaccination by use of a peptide library spanning the whole JEV proteome. Once a T cell epitope is identified, its cross-reactivity against DENV variant peptides was also measured. Finally, the identified T cell responses and their cross-reactivity with DENV peptides were assessed in PBMC collected before vaccination in order to determine if a T cell epitope response was present before vaccination or was a *de novo* response mounted following LAJV administration.

A diagram of the work performed by me, on stored samples, during the period 2015-2017, and by others colleagues in 2012-2013 is shown in figure 41.

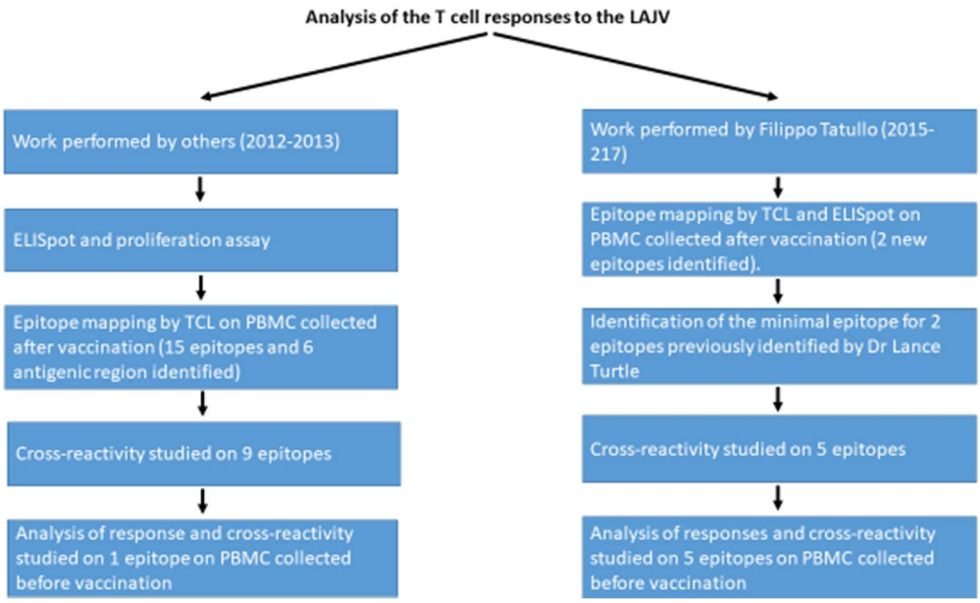


Figure 41. Flow diagram of the T cell experiments performed.

Results

Further analysis of the T cell response measured by IFNγ-ELISpot

NS3 and NS5 have been described as the main immunodominant proteins for T cell responses in flaviviruses[292]. To test the hypothesis that T cell responses to the LAJV are mainly directed against NS3 and NS5 proteins, I re-analysed the ELISpot data previously obtained by other colleagues stratifying the responses per number of volunteers that show a response to a specific protein or per average number of spots per million PBMC for each protein. Responses against NS1, E, NS3 and NS5 were the most frequent with 10, 9, 9 and 9 participants responding to these proteins respectively (figure 42). However, no difference could be observed among the JEV proteins in terms of magnitude (average number of spots per protein) of the responses (figure 43). These data are in agreement with previous works on other flavivirus vaccines which demonstrated T cell responses across all proteins[286 287].

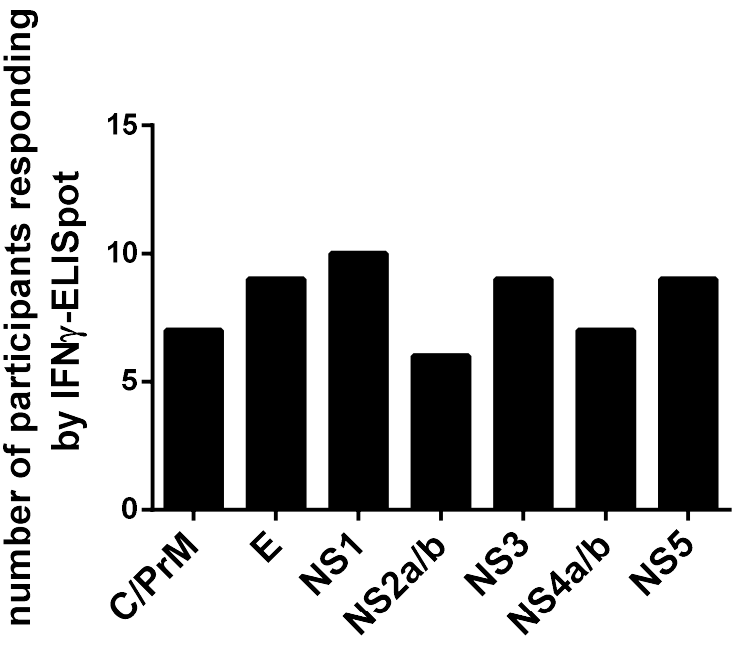


Figure 42. Number of participants responding to peptide pools stimulation by IFNγ-ELISpot per protein.

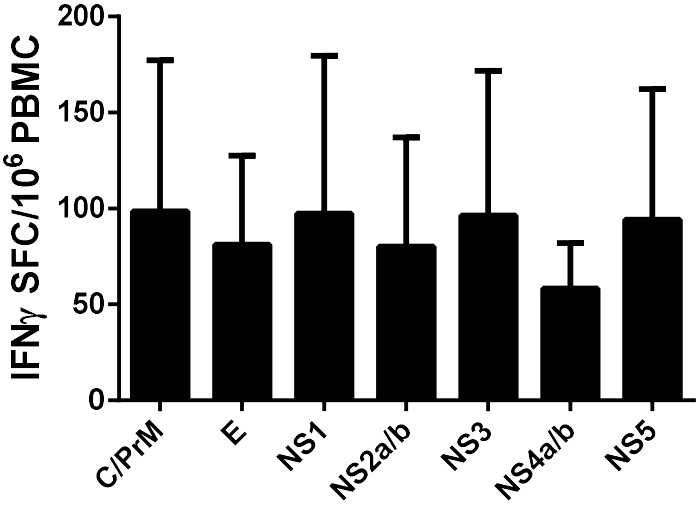


Figure 43. T cell response against each JEV protein following LAJV.

Average number of spots per response against JEV proteins measured by IFNγ-ELISpot from all participants with error bars indicating standard deviation.

To assess the correlation between T cell and antibody response following vaccination, the participants were divided into those who mounted NAb response to JEV and those

who did not (chapter 2, table 18). A comparison of the T cell response among responders (n=3) and non responders (n=13) was performed. Interestingly, no significant difference was observed indicating that despite not producing NAb specific to JEV, most of the participants were able to mount a T cell response (figure 44 and table 33). One possibility is that T cell responses in the non responders are due to DENV cross-reactive T cells that expanded following vaccination. To test this hypothesis, non responders were stratified according to their DENV serological status. However, no difference was observed among the non responding DENV seropositive (n=9) and seronegative (n=4) individuals, confirming that some participants were able to mount a T cell response without producing NAb following LAJV vaccination (figure 45). Overall, considering NAb and T cell response only 2 participants out of 15 (IFNγ-ELISpot was not performed for 010c3k1) did not respond to the LAJV at all (table 33).

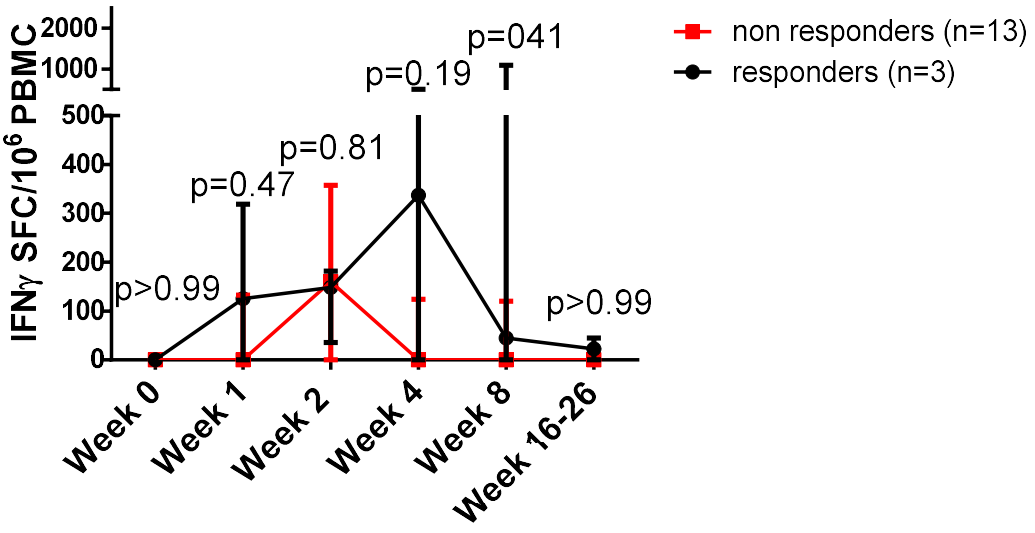


Figure 44. T cell responses among responders and non responders to LAJV by NAb.

Median of SFC / 1 million PBMC with bar indicating the interquartile range. Response was measured by IFNγ-ELISpot against all peptide pools individually in responders and non responders. p value measured by Mann-Whitney test is shown for each time point.

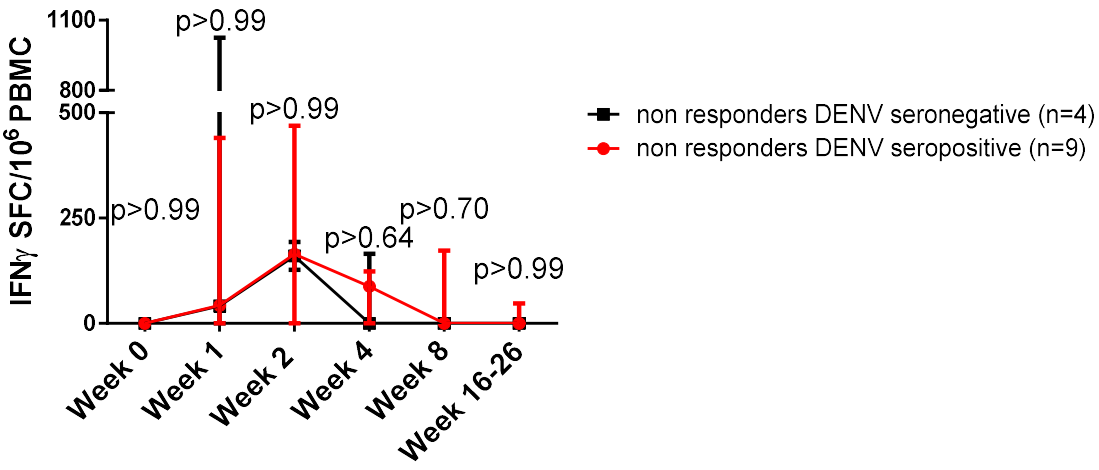


Figure 45. T cell responses among non responders (by NAb) DENV seropositives and seronegatives.

Median of SFC / 1 million PBMC with bar indicating the interquartile range. Response was measured by IFNγ-ELISpot against all peptide pools individually in non responders DENV seropositive and seronegative participants. p value measured by Mann-Whitney test is shown for each time point.

Although all participants were vaccinated with the LAJV, some participants showed a higher NAb response against DENV in contrast to a NAb response to JEV which was lower or null. This observation consisted in the phenomenon of OAS. To test the hypothesis that T cell response specific to JEV may be hindered in volunteers with OAS, I stratified the participants according to the observation of the phenomenon of OAS in the NAb response. Although the sample size was small, no statistical difference was found among participants who developed OAS (n=3) and who did not (n=9) (DENV seronegative participants were not included in this analysis) indicating that OAS observed for antibody response was unrelated to the T cell responses specific to JEV (figure 46).

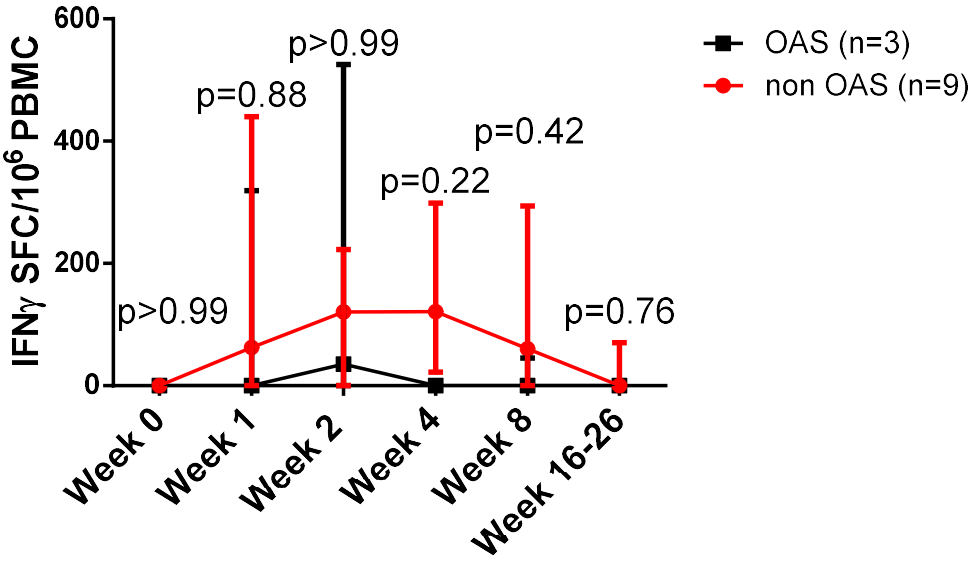


Figure 46. T cell responses following LAJV administration stratified per OAS.

Median of SFC / 1 million PBMC with bar indicating the interquartile range. Response was measured by IFNγ-ELISpot against all peptide pools individually in participants who developed OAS and who did not (DENV seronegatives were excluded). p value measured by Mann-Whitney test is shown for each time point.

Table 33. T cell and NAb response and OAS following LAJV

Participant	Response following LAJV		
	IFNγ-ELISpot	NAb	OAS
005c3k1	Yes	Yes	Yes
001c1k1	Yes	Yes	No
023c1k1	No	Yes	No
015c3k1	Yes	No	No
023c3k1	Yes	No	No
020c1k1	Yes	No	No
008c3k1	No	No	No
019c3k1	No	No	Yes
007c1k1	Yes	No	Yes
017c1k1	Yes	No	No
022c3k1	Yes	No	No
018c1k1	Yes	No	No
012c3k1	Yes	No	No
020c3k1	Yes	No	No
004c1k1	Yes	No	No
010c3k1	NA	No	No

T cell epitope mapping

T cell responses in secondary heterologous DENV infection or in secondary flavivirus infection are mostly towards cross-reactive epitopes[255,293]. To test the hypothesis that T cell responses against JEV but cross-reactive to DENV were more frequent in DENV exposed participants, T cell epitope mapping and cross-reactivity was studied. Although ELISpot is able to identify T cell responses, it cannot be used to study cross-reactive T cell responses among JEV and DENV peptide variants.

The first step to study cross-reactivity among T cell epitopes is to identify T cell responses to JEV. To identify and characterize the T cell responses, short-term T cell lines (TCL) were expanded *in vitro* using JEV peptides. T cell epitope mapping was

preferentially performed by using PBMC collected at the time point that showed the highest response by ELISpot to the peptide pools.

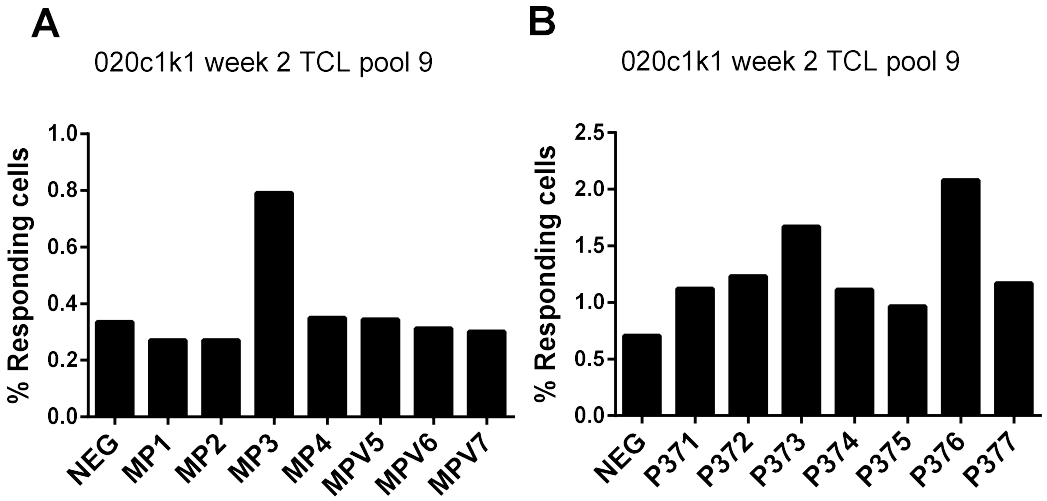
The initial strategy used consisted of expansion of a TCL using a pool of peptides. Epitope mapping was performed in two steps: firstly, the TCL was stimulated with deconvoluted mini-pools (MP) of 6-10 peptides and tested by intracellular cytokine staining (ICS), followed by another round of stimulation of the TCL using the peptides of the responding MP.

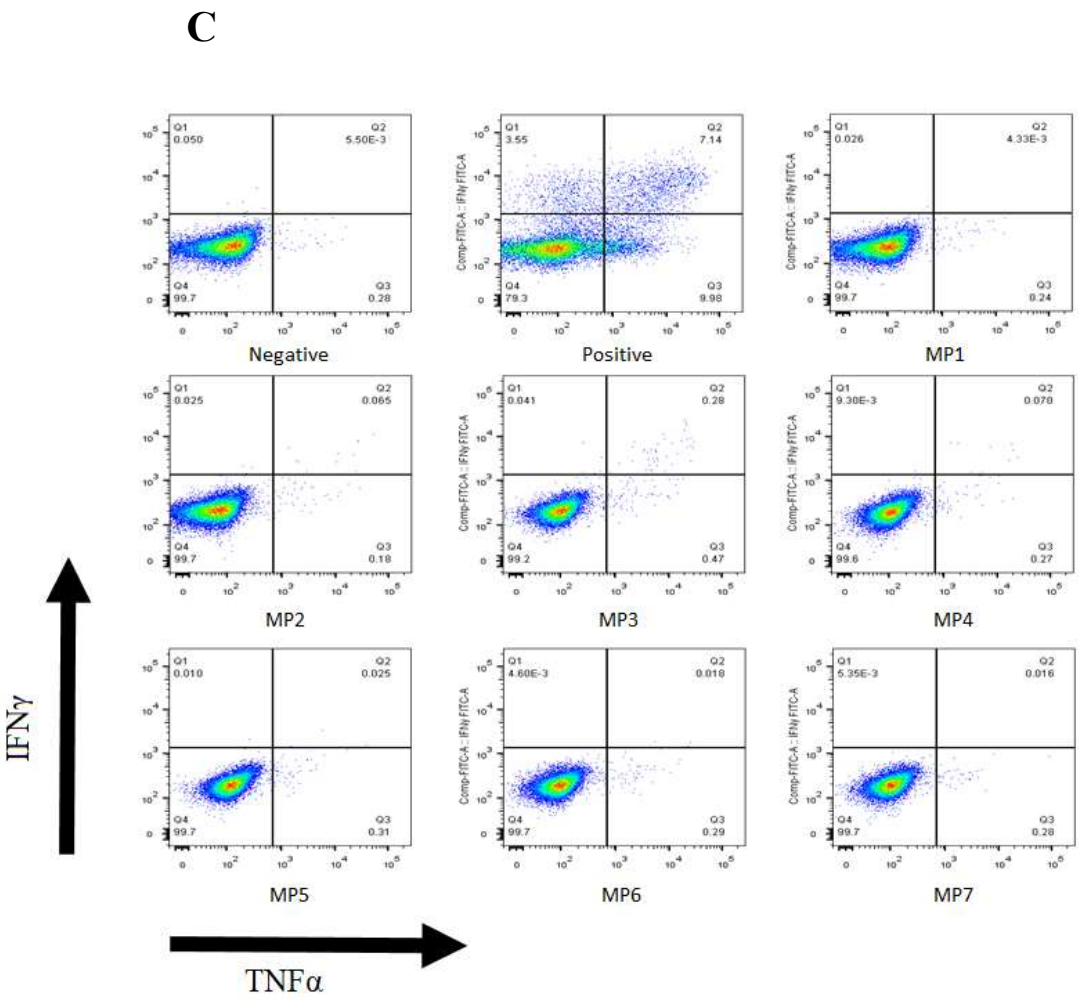
Figure 46 illustrates an example of the epitope mapping strategy using stored PBMC from participant 020c1k1. This participant responded to pool 9 (NS5, 2526-2841) by ELISpot (62 SFC/10^6 PBMC) at week 2. A TCL was expanded against pool 9 from PBMC collected at week 2. Following 7 days of expansion, the TCL was stimulated with MP of pool 9 and response identified by ICS. A small, but clear response was detected in 0.79% of $CD3^+CD8^+$ cells stimulated with MP3 (NS5, 2602-2659). In the second step to map the epitope, the TCL was stimulated with the peptides that constituted MP3 (P371-P377) (table 34):

Table 34. Peptides composing MP3 of pool 9.

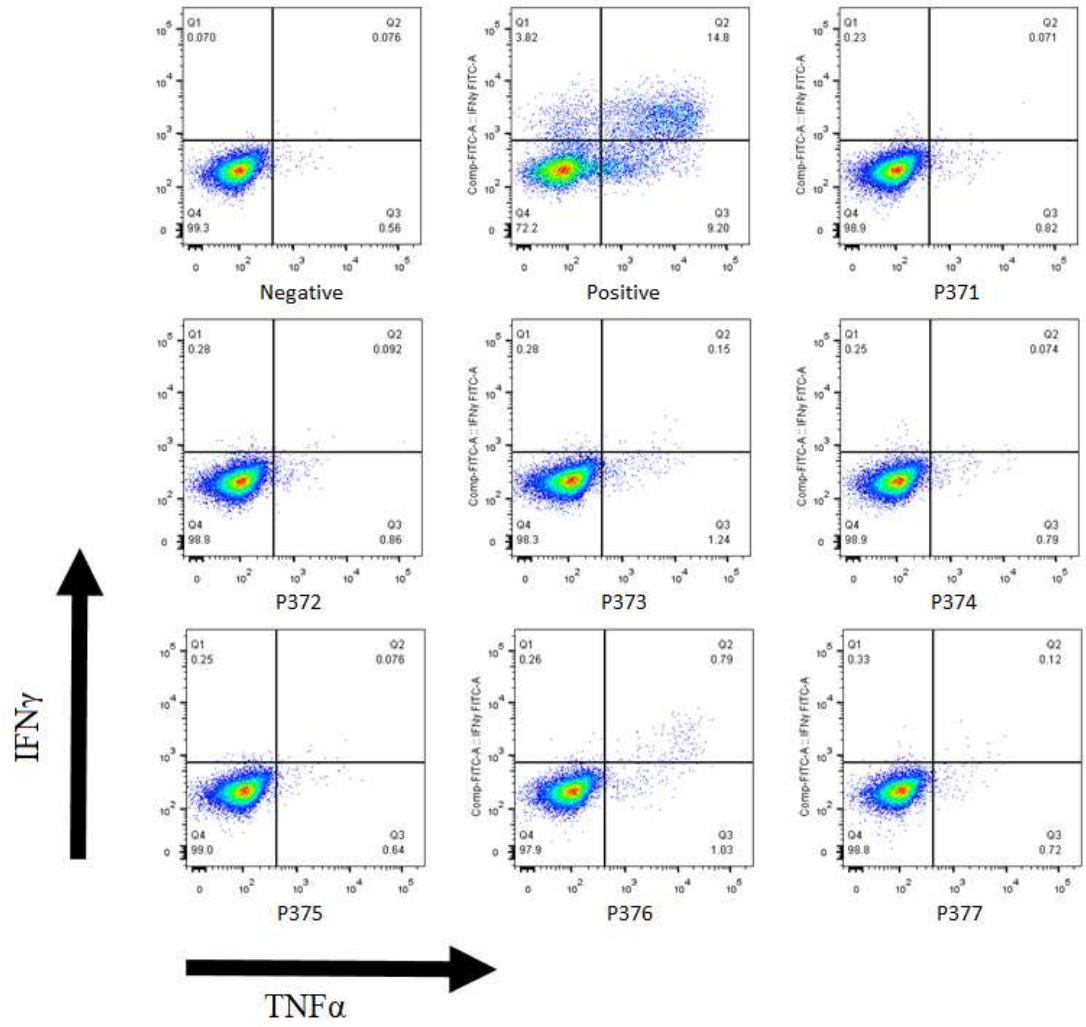
Peptide	Sequence	Protein	AA position
371	GKVIDLGCGRGGWSYY	NS5	2602-2617
372	GCGRGGWSYYAATLKKV	NS5	2608-2624
373	SYYAATLKKVQEVRGYTK	NS5	2615-2632
374	KVQEVRGYTKGGAGH	NS5	2623-2637
375	RGYTKGGAGHEEPMLM	NS5	2628-2643
376	GAGHEEPMLMQSYGWNLV	NS5	2634-2651
377	LMQSYGWNLVSLKSGVDV	NS5	2642-2659

These data identified the antigenic peptide as P376 (GAGHEEPMLMQSYGWNLV, NS5, 2634-2651) with a percentage of responding cells of 2.08%.


A
020c1k1 week 2 TCL pool 9
% Responding cells
1.0
0.8
0.6
0.4
0.2
0.0
NEG
MP1
MP2
MP3
MP4
MPV5
MPV6
MPV7
B
020c1k1 week 2 TCL pool 9
% Responding cells
2.5
2.0
1.5
1.0
0.5
0.0
NEG
P371
P372
P373
P374
P375
P376
P377


C
Negative
Positive
MP1
MP2
MP3
MP4
MP5
MP6
MP7
IFNγ
TNFα

D



D

Figure 47. Epitope mapping strategy.

TCL expanded from PBMC collected at week 2 from 020c1k1. In the first step the TCL was stimulated with MPs of pool 9 for 6 hours and T cell response measured by ICS (A and C) (cells gated on lymphocytes/singlets/live/CD3$^+$/CD8$^+$). In the second step, the TCL was stimulated with the peptides of MP3 for 6 hours and response measured by ICS identifying the responding epitope P376, GAGHEEPMLMQSYGWNLV, NS5, 2634-2651 (B and D).

Epitope mapping was initially performed by Dr Lance Turtle who identified 21 T cell epitopes (15 peptides (9-23mer) and 6 antigenic regions (46-84AA)) from 6 participants[158].

I expanded this work by growing 22 TCLs from 9 participants as illustrated in table 35. However, T cell expansion followed by epitope mapping was successfully achieved for only 2 volunteers which lead to the identification of a total of 2 epitopes both from $CD3^+CD8^+$ cells: GAGHEEPMLMQSYGWNLV (NS5, 2634-2651) from 020c1k1 and NVKDTACLAKAYAQMWLL (NS5, 2634-2651) from 007c1k1 (table 36).

TCL expansion was also achieved for another 3 epitopes which were previously identified by Dr Lance Turtle all from $CD3^+CD8^+$ cells: ALRGLPVRY (NS3, 1739-1747) from 001c1k1, TAVLAPTRVVAAEMAEVL (NS3, 1723-1740) from 012c3k1 and IEGASGATWVDLVLEGDSCLTIM (E, 306-328) from 020c1k1 (table 36)[158]. More cells were available for these volunteers, therefore these TCL expansions were carried out to confirm previously obtained data and better characterise these T cell responses.

Table 35. TCL expanded and epitopes identified

Subject ID	Week tested	Stimulus for expansion	Epitope identified	T cell subset
001c1k1	Week 4	RVPNYNLFVMDEAHF, NS3, 1779-1793 DFHFIDDPGVPWKVWVLR, NS2b, 1457-1474	None	NA
001c1k1	Week 2	ALRGLPVRY, NS3, 1739-1747	ALRGLPVRY, NS3, 1739-1747	CD8
001c1k1	Week 8	NS3, 1810-2126	None	NA
004c1k1	Week 2	NS5, 2832-3130	None	NA
004c1k1	Week 4	NS5, 2832-3130	None	NA
018c1k1	Week 4	NS3, 1500-1819	None	NA
018c1k1	Week 4	NS3, 1500-1819	None	NA
018c1k1	Week 1	NS3, 1500-1819	None	NA
020c1k1	Week 1	IEGASGATWVDLVLE, E, 306-320 GATWVDLVLEGDSCLTIM, E, 311-328	GATWVDLVL, E, 311-319	CD8
020c1k1	Week 2	NS3, 1810-2126	None	NA
020c1k1	Week 4	NS3, 1810-2126	None	NA
020c1k1	Week 2	NS5, 2526-2841	NS5, 2602-2659	CD8
020c1k1	Week 4	NS5, 2526-2841	GAGHEEPMLMQSYGWNLV, NS5, 2634-2651	CD8

020c3k1	Week 2	E, 538-799	None	NA
023c3k1	Week 4	E, 294-547	None	NA
007c1k1	Week 2	NS5, 3121-3432	NS5, 3247-3296	CD8
007c1k1	Week 2	NS5, 3121-3432	NVKDTACLAKAYAQMWLL, NS5, 3279-3296	CD8
007c1k1	Week 4	NS5, 3121-3432	NVKDTACLAKAYAQMWLL, NS5, 3279-3296	CD8
012c3k1	Week 1	NS2a/b, 1145-1509	None	NA
012c3k1	Week 1	NS5, 3121-3432	None	NA
012c3k1	Week 1	TAVLAPTRVVAAEMAEVL, NS3, 1723-1740	APTRVVAAEM, NS3, 1727-1736	CD8
017c1k1	Week 1	WLFENGEERVTRMAI, NS5, 3178-3192	None	NA

As PBMC were stored in the liquid nitrogen for 3-5 years, proliferation capacity of the cells could have been compromised making difficult identification of T cell epitopes by expansion of TCL. For this reason, an alternative approach, IFNγ-ELISpot directly on thawed cells without *in vitro* expansion, was used to try to map other T cell epitopes. Despite resting the PBMC in R10 overnight after having thawed them before stimulating them with peptides[294], all participants showed high background on stimulation with the DMSO control. A spot count at least three times that of the negative control was considered as positive response. Despite several attempts, on different participants and time points, no T cell epitope could be identified.

Overall, combining the results obtained by me and other colleagues on this cohort of participants, 17 T cell epitopes (CD4 n=9, CD8 n=7, not determined n=1) from a total of 6 volunteers were identified. Interestingly, 8 responses were identified from the volunteer 019c3k1. Five responses were identified against the prM and NS3, 3 against NS1, 2 against NS5 and only 1 against E and NS2b proteins (figure 48).

Table 36. Summary of the epitopes analysed in this study

ID	ELISpot positive before vaccination	ELISpot positive after vaccination	Epitope identified	T cell epitope identified before vaccination by TCL	T cell epitope identified after vaccination by TCL	Cross-reactive response	Proliferation Assay positive before vaccination	Previously identified by Dr Lance Turtle
005c3k1	No	Yes	NA	NA	NA	NA	No	NA
001c1k1	Yes	Yes	ALRGLPVRY, NS3, 1739-1747	Yes	Yes	Yes	Yes	Yes
023c1k1	No	Yes	NA	NA	NA	NA	Yes	NA
015c3k1	No	No	NA	NA	NA	NA	Yes	NA
023c3k1	No	Yes	NA	NA	NA	NA	NT	NA
020c1k1	No	Yes	GATWVDLVL, E, 311-319	Yes	Yes	Yes	Yes	Yes
			GAGHEEPMLMQSYGWNLV, NS5, 2634-2651	No	Yes	No		No
008c3k1	No	No	NA	NA	NA	NA	Yes	NA
019c3k1	No	No	NA	NA	NA	NA	Yes	NA

007c1k1	No	Yes	NVKDTACLAKAYAQMWLL, NS5, 3279-3296	Yes	Yes	Yes	No	No
017c1k1	No	Yes	NA	NA	NA	NA	Yes	NA
022c3k1	No	Yes	NA	NA	NA	NA	NT	NA
018c1k1	No	Yes	NA	NA	NA	NA	Yes	NA
012C3k1	No	Yes	APTRVVAAEM, NS3, 1727-1736	No	Yes	Yes	Yes	Yes
020c3k1	No	Yes	NA	NA	NA	NA	No	NA
004c1k1	No	Yes	NA	NA	NA	NA	No	NA
010c3k1	No	NT	NA	NA	NA	NA	NT	NA
Total	**1/16**	**12/15**	**NA**	**3/5**	**5**	**4/5**	**9/13**	**3/5**

NT= not tested

NA= not applicable

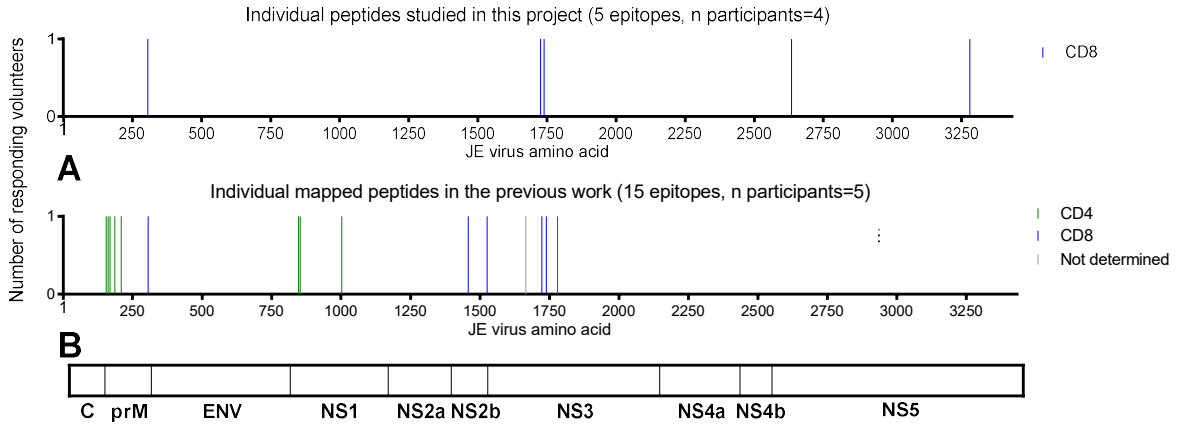


Figure 48. Summary of identified T cell epitopes.

Location of the epitopes studied in this project (A) and in the previously published work (B). Figure edited from Turtle et al.[158]

As more cells were available for the participants 020c1k1 and 012c3k1, the minimal epitopes corresponding to two peptides previously identified by others from these participants were determined. The previously identified peptides were: IEGASGATWVDLVLE (Envelope (E), 306-320) and GATWVDLVLEGDSCLTIM (E, 311-328) detected from the participant 020c1k1 and peptide TAVLAPTRVVAAEMAEAL (NS3, 1723-1740) identified from 012c3k1. Both of these epitopes were detected from CD8 T cells. Fine mapping was performed by growing the TCL with the peptide epitope and stimulating it with different truncated variants of the peptide and analysing which variant was giving the highest response. Truncated variants were designed by Dr Lance Turtle by an epitope binding prediction software (iedb.org) using the HLA I molecules identified from these participants. HLA typing was performed by Rotary TTK blood bank, Bangalore by polymerase chain reaction.

A TCL expanded with IEGASGATWVDLVLE and GATWVDLVLEGDSCLTIM from PBMC collected from 020c1k1 at week 1 was stimulated with the truncated peptides: ATWVDLVL, GATWVDLVL, GASGATWVDL and VLEGDSCLTI. Responses were observed only when the first two peptides (1.21% and 1.36% responding cells for ATWVDLVL and GATWVDLVL respectively) were used to stimulate the TCL (figure 49). The peptide GATWVDLVL was considered to be the minimal epitope as a slightly higher response was obtained. Using a similar strategy, a TCL expanded with TAVLAPTRVVAAEMAEAL from PBMC collected at week 2 from 012c3k1 identified the peptide APTRVVAAEM as the minimal epitope.

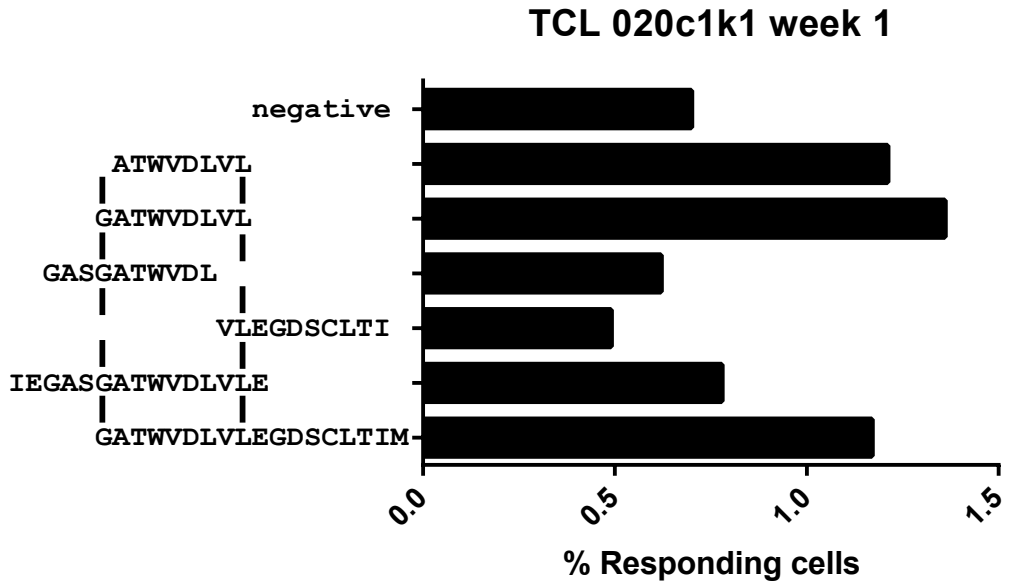


Figure 49. Example of identification of T cell minimal epitope.

TCL expanded with IEGASGATWVDLVLE (E, 306-320) and GATWVDLVLEGDSCLTIM (E, 311-328) from PBMC collected at week 1 from 020c1k1. The minimal T cell epitope was identified by stimulation of the TCL with the peptide variants indicated, followed by measurement of the response by ICS. Data are percentage of $CD8^+$ T cells staining for IFNγ or TNFα in any combination.

Cross-reactivity of the T cell epitopes

As the 4 DENV serotypes co-circulate with JEV in India and majority of the participants were shown to be DENV exposed by the presence of NAb (chapter II, table 19), cross-reactivity of the T cell epitopes identified was also studied. Expansion of TCLs could be achieved for only few epitopes and participants therefore it was not possible to test the hypothesis that cross-reactive epitopes were more frequent in DENV exposed participants. T cell cross-reactivity among flavivirus is known, therefore I tested the hypothesis that the live attenuated JEV vaccine would have raised DENV cross-reactive T cell responses. For this work, peptide libraries of the E, NS1, NS3 and NS5 proteins of DENV 1, 3 and 4, and a full length library for DENV2 were obtained from Biodefense and Emerging Infection resources (National Institutes of Health).

Cross-reactivity was studied by stimulating the short-term TCL expanded with the JEV peptide individually with the corresponding peptides of the 4 DENV serotypes and the JEV peptide. As before, the cross-reactivity index (CRI) was defined as the percentage of responding cells

to the DENV variant peptides divided by the percentage of responding cells to the JEV peptide, after background subtraction[157]. Therefore, a value of 1 indicates a response of equal magnitude to JEV, a response >1 indicated a DENV response higher than JEV whereas a response <1 indicated a not fully cross-reactive response among JEV and DENV.

I studied the cross-reactivity on a total of 5 epitopes for which a TCL could be expanded. These epitopes included the peptides NVKDTACLAKAYAQMWLL, GATWVDLVL, GAGHEEPMLMQSYGWNLV, APTRVVAAEM and an epitope previously identified and described by Dr Lance Turtle from the participant 001c1k1: ALRGLPVRY (NS3, 1739-1747)[158].

Additionally, the possibility of degranulation (killing) following peptide stimulation was studied by staining for CD107a. Cytotoxicity involves the release of pre-formed lytic granules, containing perforin and granzymes, toward the target cell. This process exposes to the cell membrane numerous lysosomal-associated membrane glycoproteins (LAMPs) such as CD107a. Thus, labelling cells with antibodies specific to CD107a (which is added during the assay set up) identify degranulating cells[257].

Finally, each of these epitopes and their cross-reactivity were studied on PBMC collected before vaccination. These experiments were crucial to establish which response was already present before vaccination and which one was induced by the administration of the LAJV.

Epitopes identified from 020c1k1

Two epitopes were identified from the participant 020c1k1: GATWVDLVL (E, 311-319) and GAGHEEPMLMQSYGWNLV (NS5, 2634-2651).

Cross-reactivity for GATWVDLVL was previously studied on PBMC collected after vaccination (week 1) by Dr Lance Turtle by using the non-truncated JEV peptide (GATWVDLVLEGDSCLTIM) instead of the minimal epitope and high degree of cross-reactivity was observed for DENV1/3[158]. I confirmed and expanded previous data for this epitope. TCL expanded at week 1 with GATWVDLVL indicated high degree of cross-reactivity to DENV1/3 (CRI 0.78) and to a lesser extent with DENV4 (CRI 0.28) (figure 50 and 51). Response to DENV4 was not previously observed by Dr Lance Turtle for this epitope on this participant. This may be due to better expansion promoted by the minimal epitope peptide. Moreover, higher level of degranulation was observed when the TCL was stimulated with the DENV1/3 (1.28% of $CD3^+CD8^+$ cells) and DENV4 (0.35% of $CD3^+CD8^+$ cells) in comparison to the JEV peptide (0.23% of $CD3^+CD8^+$ cells) (figure 50).

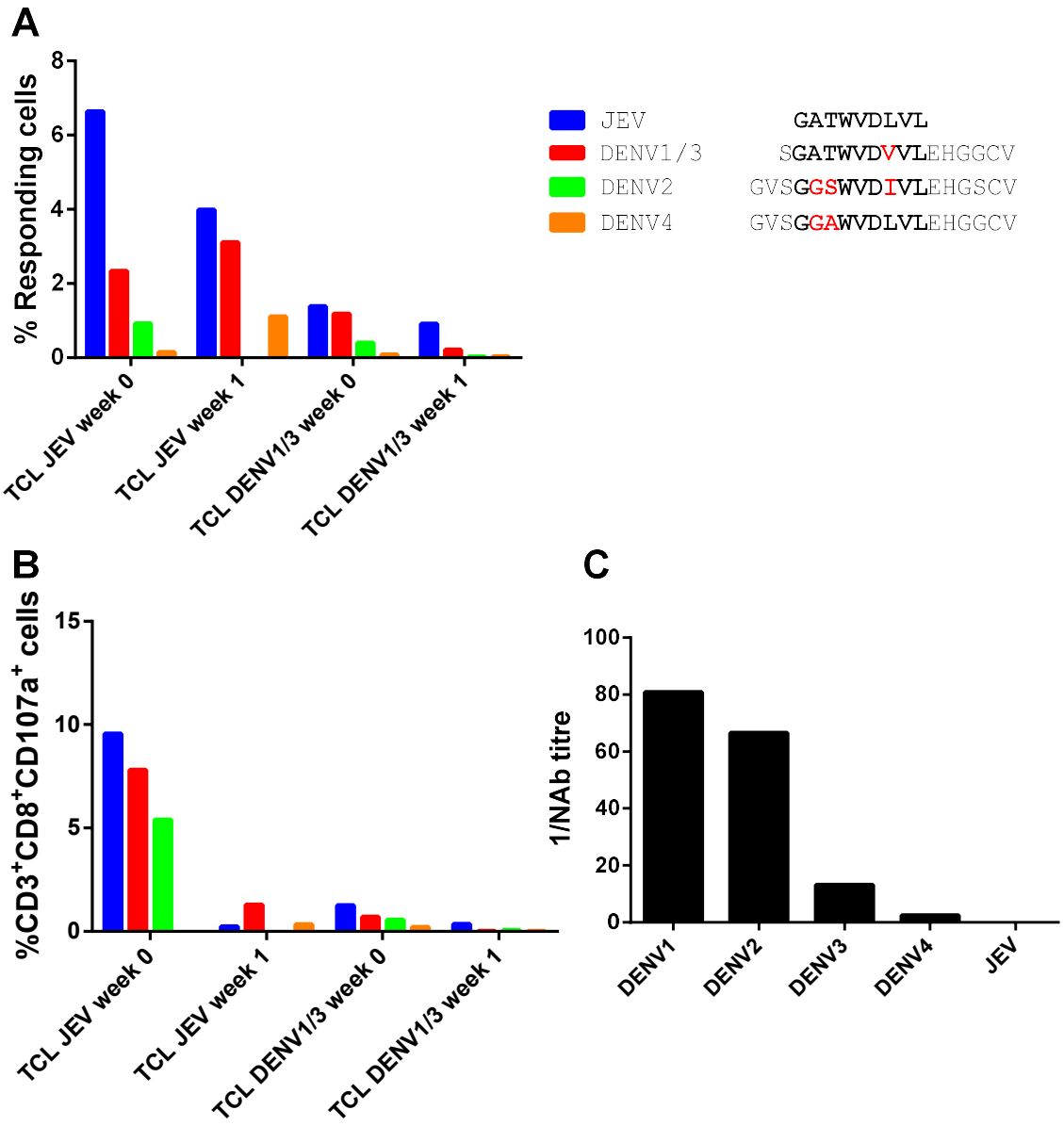


Figure 50. Cross-reactivity of GATWVDLVL in 020c1k1.

TCL expanded with GATWVDLVL (JEV) and SGATWVDVVLEHGGCV (DENV1/3) from PBMC collected at week 0 and 1 from 020c1k1. Each TCL was stimulated with all DENV serotype variants and JEV peptide. Data shown are percentages of $CD3^+CD8^+$ T cells stained for IFNγ or TNFα in any combination (A) and CD107a (B). Reciprocal NAb titre at baseline measured by PRNT against all DENV serotypes and JEV are also shown (C).

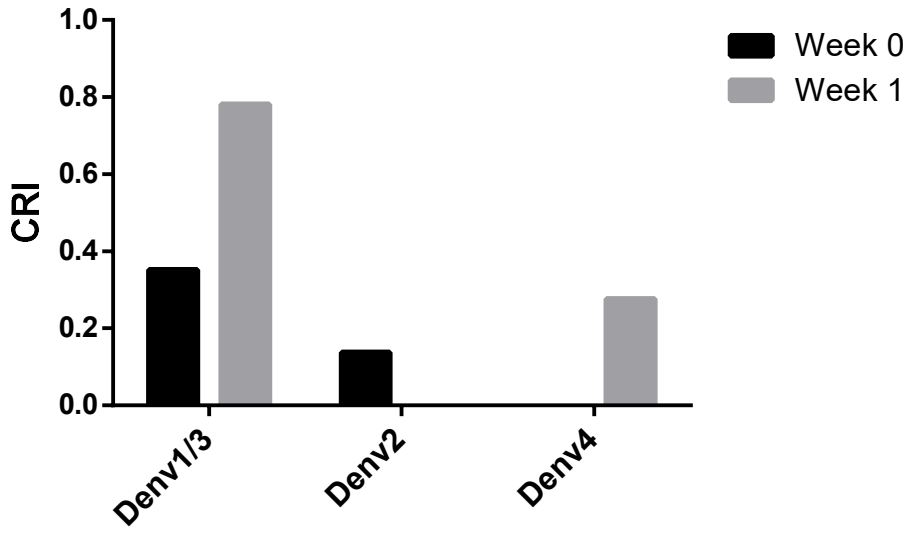


Figure 51. Cross-reactivity index of GATWVDLVL in 020c1k1.
TCL expanded with GATWVDLVL (JEV) from PBMC collected at week 0 and 1 from 020c1k1 was stimulated with JEV and DENV peptide variants and percentage of responding cells measured by ICS. CRI was measured as ratio between the percentage of responding cells to DENV variants and the JEV peptide.

Interestingly, when a TCL was expanded at week 1 with DENV1/3 variant, response (0.91% of responding cells) and degranulation (0.36% of $CD3^+CD8^+$ cells) was detected only against the JEV peptide (figure 50), possibly reflecting the increased efficiency of the ICS assay using the minimal peptide.

As this response was cross-reactive and this participant had NAb against DENV1, 2 and 3 at baseline, TCLs were expanded with the JEV and DENV1/3 peptides from PBMC collected before vaccination to understand if this response was present before vaccination or it was a *de novo* response raised following LAJV administration. Both TCLs showed an expansion indicating that this response was elicited following a previous flavivirus infection. Responses to DENV1/3, 2 and JEV were respectively of 2.34% (CRI of 0.35), 0.92% (CRI of 0.14) and 6.64% of responding cells when the TCL was expanded with JEV peptide. Similarly, responses to DENV1/3, 2 and JEV were observed when the TCL was expanded with DENV1/3 peptide with respectively 1.17%, 0.40%, and 1.38% responding cells. Overall, whereas DENV2 was showing a similar level of CRI before and after vaccination, the CRI increased by 2.22-fold following LAJV administration for DENV1/3, indicating that perhaps not all DENV

responding cell populations expanded equally. Finally, a cross-reactive response to DENV4 was not detected before vaccination but was observed after vaccination. A possibility to explain this is that *de novo* T cells specific to JEV and cross-reactive to DENV4 may have raised following vaccination. TCL expanded with JEV at week 0 showed higher degranulation after stimulation with JEV (9.6% of $CD3^+CD8^+$ cells) followed by DENV1/3 (7.8% of $CD3^+CD8^+$ cells) and DENV2 (5.4% of $CD3^+CD8^+$ cells) peptides. A similar pattern was observed when a TCL was expanded on the same time point with DENV1/3 peptide. Higher percentage of degranulation was observed when the TCL was stimulated with the JEV peptide (1.26% of $CD3^+CD8^+$ cells) in comparison to DENV1/3 and 2 which showed a percentage of degranulation of $CD3^+CD8^+$ cells of 0.71% and 0.56% respectively (figure 50).

Conversely to GATWVDLVL (E, 311-319), GAGHEEPMLMQSYGWNLV (NS5, 2634-2651) epitope did not cross-react with any DENV serotype and could not be expanded before vaccination indicating that this was an epitope raised following vaccination with LAJV.

Taking together these results demonstrated that previous DENV exposure does not hinder the development of new T cell responses with cytotoxic function.

Epitope identified from 007c1k1

The peptide NVKDTACLAKAYAQMWLL was identified from 007c1k1. TCL expanded with JEV at week 2 demonstrated that this epitope cross-reacted with all DENV variants although the higher degree of cross-reactivity was observed against DENV1/2 (which were sharing the same AA sequence for this epitope) and DENV4. The percentage of responding cells detected for DENV1/2, 3, 4 and JEV were respectively of 0.76% (CRI of 0.62), 0.48% (CRI of 0.39), 0.71% (CRI of 0.58) and 1.22% (figure 52 and 53). Interestingly, response to this epitope was detected before vaccination indicating that it was not vaccine induced. As this participant was DENV seropositive and JEV seronegative at baseline it is likely that the response to this epitope was raised initially due to a DENV infection. No cross-reactive response against DENV3 and 4 were detected on a TCL expanded with JEV peptide at baseline whereas 0.35% (CRI of 0.69) and 0.51% of responding cells were detected for DENV1/2 and JEV respectively (figure 52 and 53). A TCL expanded using the DENV1/2 variant from PBMC collected before vaccination confirmed the high degree of cross-reactivity among DENV1/2 (1.4% responding cells) and the JEV variant (1.35% responding cells). In addition, a small cross-reactive response was also detected when the TCL was stimulated with DENV4 peptide variant (0.32% responding cells) (figure 52).

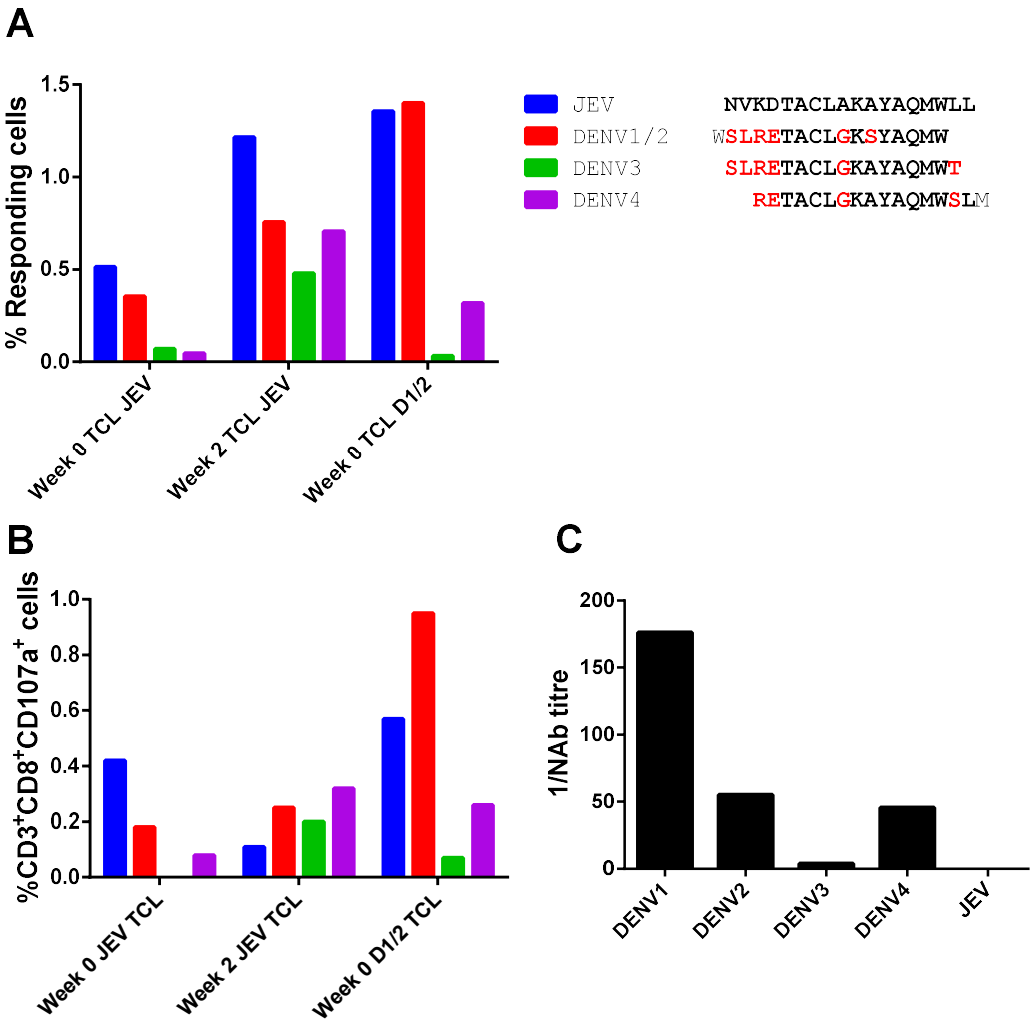


Figure 52. Cross-reactivity of NVKDTACLAKAYAQMWLL in 007c1k1.

TCL expanded with NVKDTACLAKAYAQMWLL (JEV) from PBMC collected at week 0 and 2 and with WSLRETACLGKSYAQMW (DENV1/2) from PBMC collected at week 0 from 007c1k1. Each TCL was stimulated with JEV and all DENV serotype variant peptides. Data are percentage of $CD3^+CD8^+$ T cells stained for IFNγ or TNFα in any combination (A) and CD107a (B). Reciprocal NAb titre at baseline measured by PRNT against all DENV serotypes and JEV are also shown (C).

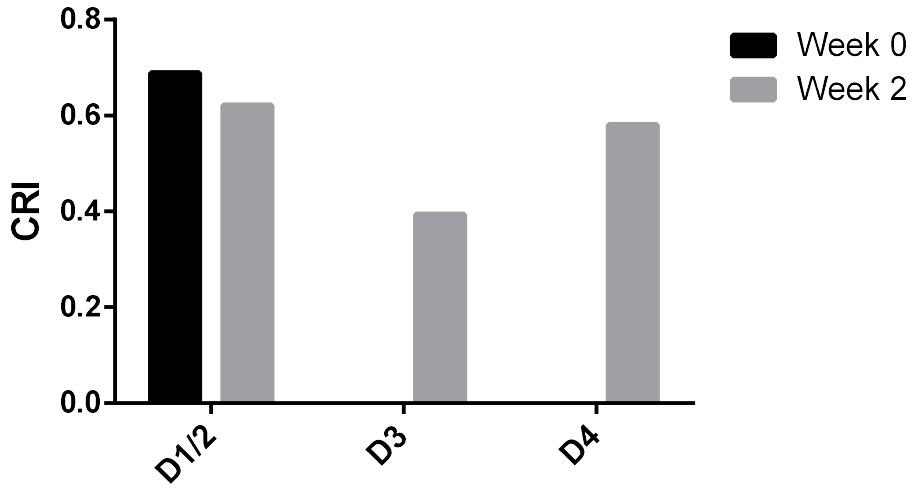


Figure 53. Cross-reactivity index of NVKDTACLAKAYAQMWLL in 007c1k1.
TCL expanded with NVKDTACLAKAYAQMWLL (JEV) from PBMC collected at week 0 and 2 from 007c1k1 were stimulated with JEV and DENV peptide variants and percentage of responding cells measured by ICS. CRI was measured as ratio between the percentage of responding cells to DENV variants and the JEV peptide.

Analysis of degranulation by staining for CD107a indicated higher levels of degranulation when the TCL expanded with JEV peptide before vaccination was stimulated with JEV peptide (0.42% of $CD3^+CD8^+CD107a^+$ cells) in comparison to the DENV variants which showed degranulation (0.18% of $CD3^+CD8^+CD107a^+$ cells) only when stimulated with DENV1/2 peptide. When a TCL was expanded with DENV1/2 before vaccination degranulation was observed mainly when stimulated with DENV1/2 and JEV (0.95% and 0.57% respectively of $CD3^+CD8^+CD107a^+$ cells) confirming the cytokines results (figure 52).

Overall, following vaccination a broadening of cross-reactivity was observed with a cross-reactive response detected also for both DENV3 and 4 which was not observed at baseline, indicating that it is likely the response was either a mixture of newly primed cells and memory cells, or memory cells that were too infrequent to be expanded from the baseline samples.

Epitopes identified from 001c1k1

In participant 001c1k1, a response to the epitope ALRGLPVRY (NS3, 1739-1747) was previously described by Dr Lance Turtle. TCLs were expanded before (with JEV wild type (WT) and DENV2/4 peptide variants) and after (week 16) (with JEV WT, JEV vaccine and DENV2/4 peptide variants) vaccination. The epitope was present before vaccination and

showed high degree of cross-reactivity among JEV WT and DENV2/4 peptides. On contrary, the vaccine variant was less efficient in expanding the TCL and showed less cross-reactivity with the other peptides although the response was similar when analysed by including the cytokine MIP-1β[158]. I repeated these experiments including the DENV1 and 3 peptide variants to confirm previous data from this volunteer. A cross-reactive T cell response with all DENV serotypes (DENV1, ALKGMPIRY; DENV2/4, ALRGLPIRY; DENV3, AMKGLPIRY) and the vaccine (VLRGLPVRY) variants was observed from a TCL expanded with ALRGLPVRY (JEV WT) at week 2. Although, the epitope was cross-reacting with all DENV serotypes, CRI was lower for DENV1 (0.18), DENV2/4 (0.41) and the vaccine variant (0.45) in contrast to DENV3 (0.84) (figure 55). When a TCL was expanded with the DENV2/4 variant at the same time point, similar levels of responses were observed among the DENV2/4 (15.77% $CD3^+CD8^+$ responding cells), DENV3 (15.16% $CD3^+CD8^+$ responding cells) and the JEV WT peptide (12.77% $CD3^+CD8^+$ responding cells) whereas DENV1 (4.35% $CD3^+CD8^+$ responding cells) and the vaccine (8.53% $CD3^+CD8^+$ responding cells) responses were smaller (figure 54). The response to this epitope was present before vaccination and TCLs to JEV WT, JEV vaccine, DENV2/4 and 3 were expanded. Cross-reactive responses could be observed for all TCLs and with any peptide to similar level except for responses to DENV1 for any TCL, to DENV3 for the TCL that was expanded with the DENV2/4 variant and to the vaccine variant on the TCL expanded with DENV3 which were showing a lower percentage of responding cells (figure 54). Furthermore, JEV WT and DENV2/4 peptides were more efficient in expanding the TCL in comparison to the vaccine and DENV3 variants. Detection of anti-DENV NAb at baseline indicated that this volunteer had been previously infected with DENV, possibly DENV 2, which was showing a higher NAb titre. Overall, analysis of the CRI showed a decrease in the level of cross-reactivity for all peptide variants following LAJV administration. As previously observed for other participants, JEV vaccination may have expanded memory as well as newly primed T cells.

Degranulation was observed for all the peptides and for all TCL expanded before and after vaccination. Before vaccination, degranulation (cytotoxic) responses were similar across all TCLs and peptides tested (figure 54) except for responses to vaccine and DENV1 on the TCL expanded with the vaccine peptide. After vaccination, conversely to what observed from the cytokine responses, percentage of $CD3^+CD8^+CD107a^+$ was similar across all peptides for both TCLs (JEV WT and DENV2).

Overall, the data obtained by me confirmed what previously observed by our group for this participant with the vaccine variant being less efficient in stimulating T cells when response was measured by production of IFNγ and/or TNFα. However, when response was measured by another marker (MIP-1β or CD107a), the vaccine peptide showed similar level of responses than other peptide variants. This may be due to lower triggering threshold for MIP-1β and CD107a.

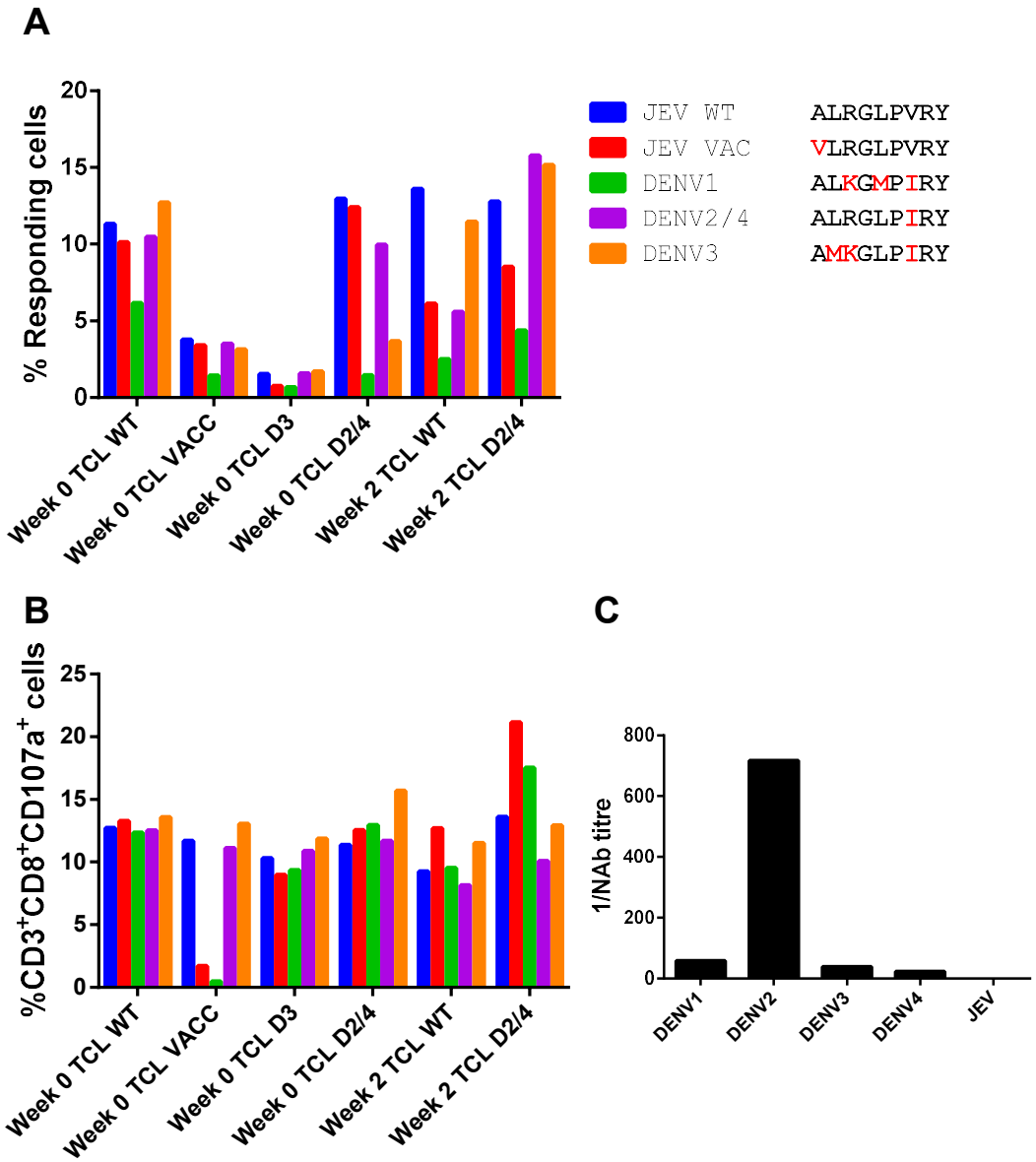


Figure 54. Cross-reactivity of ALRGLPVRY in 001c1k1.

TCL expanded with JEV WT (ALRGLPVRY) and DENV1, 2/4, 3 and vaccine variant from PBMC collected at week 0 and with JEV WT and DENV2/4 peptides from PBMC collected at week 2 from 001c1k1. Each TCL was stimulated with all DENV serotype variant and JEV (WT and vaccine) peptides. Data are presented as percentage of $CD3^+CD8^+$ T cells stained for IFNγ or TNFα in any combination (A) and CD107a (B). Reciprocal NAb titre at baseline measured by PRNT against all DENV serotypes and JEV are also shown (C).

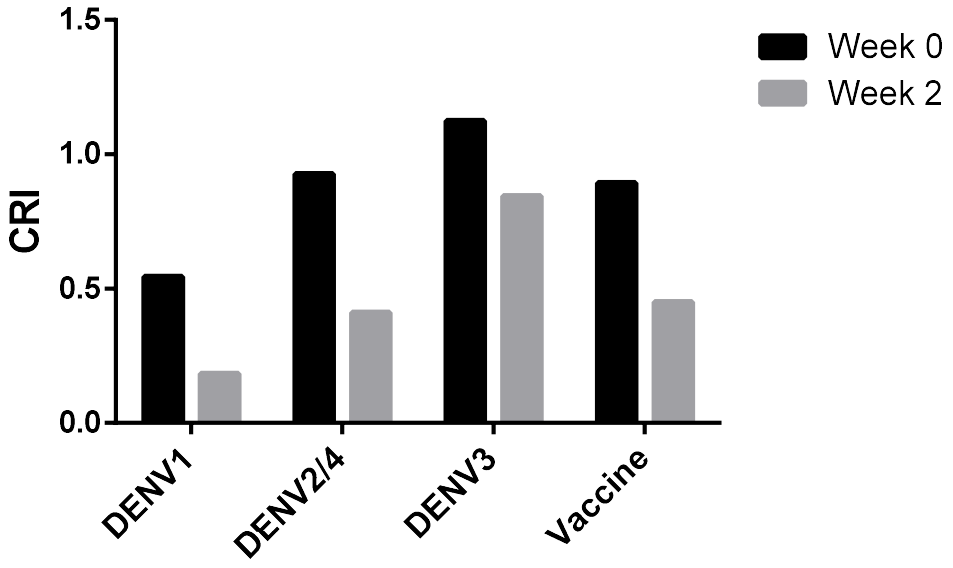


Figure 55. Cross-reactivity index of ALRGLPVRY in 001c1k1.

TCL expanded with JEV WT (ALRGLPVRY) at week 0 and 2 from 001c1k1. Each TCL was stimulated with all DENV serotype variant and JEV (WT and vaccine) peptides. CRI was measured as ratio between the percentage of responding cells to DENV variant and the JEV peptide.

Epitopes identified from 012c3k1

The epitope APTRVVAAEM was identified in 012c3k1 after vaccination. As more cells were available from this participant, a TCL expanded with this peptide from PBMC collected at week 2 was used to study cross-reactivity and identify the minimal epitope for DENV1. Truncated peptide variants were designed by an epitope binding prediction software (iedb.org) by Dr Lance Turtle using the HLA I molecules identified from this participant (Rotary TTK blood bank, Bangalore). DENV2, 3 and 4 were sharing the same AA sequence of JEV for this epitope. Responses to DENV1 variant peptides showed high degree of cross-reactivity. In addition, these data demonstrated the last AA of the epitope (methionine) was critical for the T cell response whereas the highest response was observed with LAPTRVVASEM. Interestingly, although, this participant was showing NAb to all DENV serotypes at baseline, a T cell response to APTRVVAAEM could not be detected before vaccination indicating that the response was vaccine induced (figure 56).

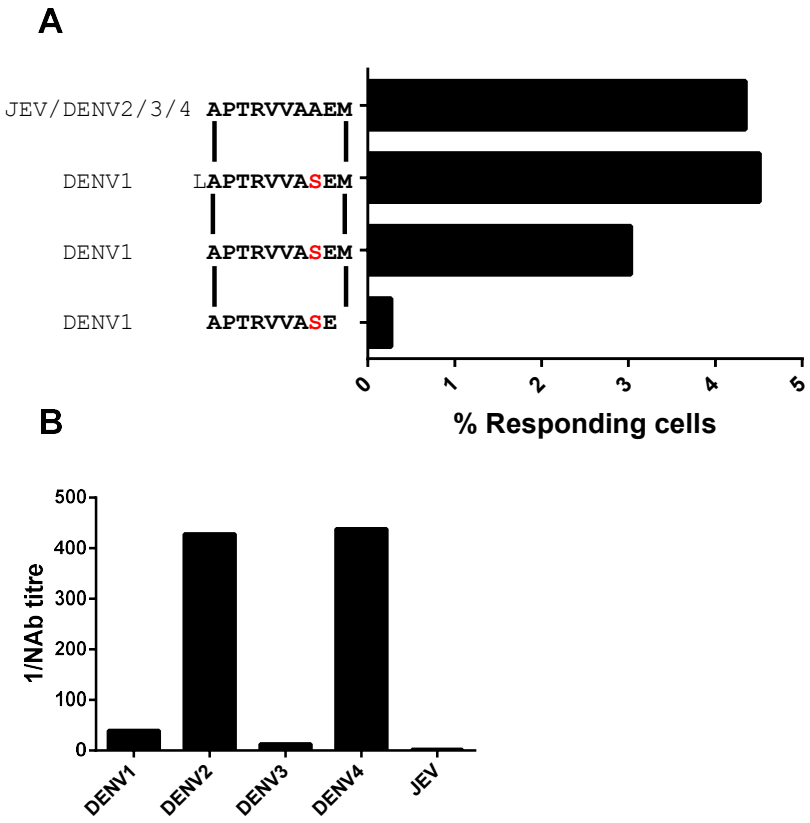


Figure 56. Cross-reactivity of APTRVVAAEM in 012c3k1.

TCL expanded with APTRVVAAEM (JEV/DENV2/3/4) from PBMC collected at week 2 from 012c3k1. Each TCL was stimulated with truncated DENV1 peptide variants and JEV/DENV2/3/4 peptide. Data are percentage of $CD8^+$ T cells stained for IFNγ or TNFα in any combination (A). Reciprocal NAb titre at baseline measured by PRNT against all DENV serotypes and JEV are also shown (B).

Summary of cross-reactivity of T cell responses

Overall, from the 13 epitopes from which cross-reactivity against DENV was tested (I tested 5 epitopes: I confirmed data for 2 epitopes previously analysed by Dr Lance Turtle and I analysed 3 epitopes which were not tested previously), 5 epitopes were cross-reactive against DENV. In particular, 4 epitopes cross-reacted against the 4 DENV serotypes, whereas 1 cross-reacted against DENV 4 only. Finally, 2 cross-reactive epitopes were observed from NS3 and 1 for E, NS1 and NS5.

As a limited number of epitopes were identified, meaningful group analysis could not be performed. However, these data demonstrated that the LAJV is able to raise cross-reactive T

cell responses. Both cross-reactive and non cross-reactive CD8 responses showed lytic function by staining for the degranulation marker CD107a. Furthermore, previous cross-reactive T cell response do not prevent generation of *de novo* T cell responses. In some cases broadening of cross-reactive responses which were present before vaccination was observed following LAJV administration indicating that the response was likely a mixture of newly primed and memory cells.

To test the hypothesis that higher NAb response to a specific serotype was related to higher cross-reactive T cell response to the same serotype following LAJV vaccination, NAb titre fold change at week 4 was plotted against the CRI fold change for each DENV serotype. In agreement with the ELISpot data, no significant correlation was identified among these values indicating no significant correlation among the antibody and T cell responses (Spearman's correlation, R=-01891, p=0.46) (figure 57). These data indicated that NAb response to a specific serotype is not dependent to the T cell response to that serotype.

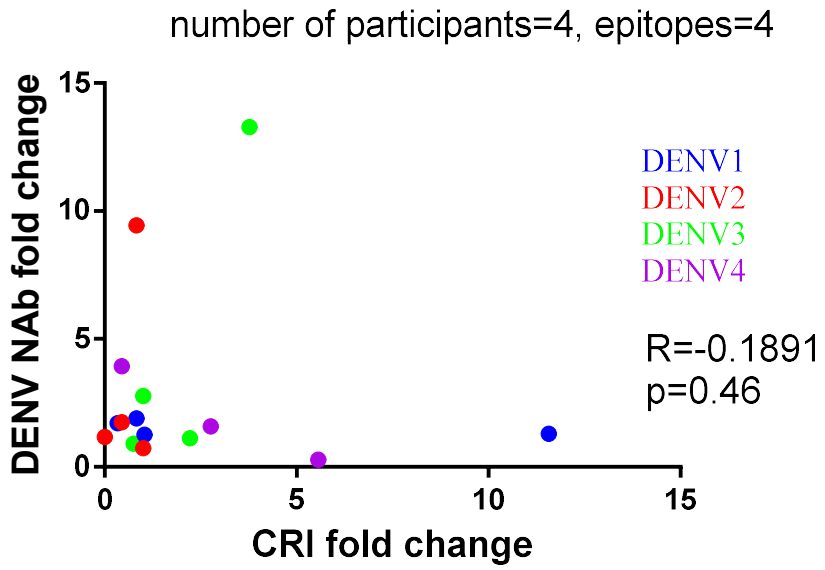


Figure 57. Correlation between the DENV NAb and CRI.

Correlation between the NAb (at week 4) and CRI fold change for each DENV serotype following LAJV.

Discussion

This work confirmed and expanded the work previously published by our group[158]. ELISpot data, which were previously published, were analysed stratifying them according to NAb response to the vaccine and the observation of the phenomenon of OAS. The PBMC collected

before vaccination were crucial as this work was carried out in a country endemic for JEV and DENV. This time point enabled the differentiation of *de novo* T cell responses raised to LAJV from anamnestic responses primed by a previous flavivirus infection.

T cell responses were identified against all proteins with E, NS1, NS3 and NS5 being the most frequent. This is in agreement with T cell responses found after vaccination with other flavivirus live attenuated vaccines such as the yellow fever and the tetravalent dengue vaccine[287,288,289].

Despite proliferative responses being detected before vaccination in some participants, IFNγ was not detected at the same time point by ELISpot. Similar findings were observed in patients sampled 6 months after recovery from JE[156] or in PBMC infected with DENV2 under ADE condition where IFNγ was blunted, contrary to proliferation and IL-10 production, which were preserved[295]. An explanation could be a difference in the incubation period (8 days vs overnight for proliferation and ELISpot assay respectively) or the type of the response as cells may have responded by producing another cytokine other than IFNγ. Finally, another possibility is the presence of central memory T cells at baseline which have high proliferating profile with production of mainly IL-2 before differentiating into effector memory and producing IFNγ[295].

Another interesting finding observed in some participants was the T cell responses in participants who did not mount a NAb response. This may represent an immune profile where protection is mediated by memory T cells[213,254,284,296]. This was previously shown with adoptive transfer and challenge studies in animal model of flavivirus infection[213,254,296].

No difference could be observed among T cell responses in participants who developed OAS in antibody response and who did not, indicating that OAS in antibody response and development of new T cell responses occur independently of each other. OAS at T cell level was observed during secondary heterologous infection with DENV with many DENV-specific T cells showing low affinity for the infecting virus leading to the speculation that OAS may delay viral clearance[202]. In dengue, OAS has been proposed as a mechanism to explained higher severity in a secondary heterologous infection[208] however some evidence indicated that OAS is more a general phenomenon that occurs in DENV infection independent of the severity of the disease[209,210]. Evidence of OAS at T cell level impairing the development of new CD8 T cell responses and promoting higher pathogenesis was found in a lymphocytic choriomeningitis virus mouse model[197].

Use of a peptide library coupled with flow cytometry allowed mapping and characterisation of epitopes for some of these T cell responses. Despite several attempts, I was able to achieve T cell expansion only for four participants. In the previous work of Dr Turtle, a good correlation was observed among *ex vivo* cross-reactive responses and expanded *in vitro* short-term TCL[157]. Therefore, analysis of T cell cross-reactivity in this work was performed on short-term TCL, which allowed the use of fewer cells. T cell cross-reactivity was observed by both cytokine production and degranulation (CD107a staining).

Initial work performed by Dr Lance Turtle on this cohort of volunteers identified both CD4 and CD8 responses. The work performed for this project, however, identified only CD8 T cell epitopes. A possible explanation could be the long-term storage of up to 5 years in the liquid nitrogen of the PBMC which may have affected the proliferative ability of T cells. Similar results were obtained by Owen *et al.* showing a decrease of CD4 T cell responses in long term cryopreserved samples[297].

I was able to expand TCL for a total of 5 epitopes from CD8 T cells and four of them showed different degrees of cross-reactivity with the 4 DENV serotype variants. Additionally, two of these epitopes (GAGHEEPMLMQSYGWNLV (NS5, 2634-2651) and APTRVVAAEM (NS3, 1727-1736)) developed after administration of the LAJV whereas the rest of them were present before vaccination (ALRGLPVRY (NS3, 1739-1747), GATWVDLVL (E, 311-319), NVKDTACLAKAYAQMWLL (NS5, 3279-3296)).

The response to the APTRVVAAEM peptide identified here was not present before vaccination and it was highly cross-reactive with all DENV variants. Considering the fact that this participant was DENV seropositive at baseline and that the epitope was cross-reactive, the possibility that the epitope was present before vaccination but failed to expand cannot be fully excluded. This epitope has been previously identified in healthy JE exposed individuals[157] and DENV infected people. Additionally this antigenic region was described to bind both HLA class I and II molecules[292,298]. In this project, APTRVVAAEM peptide was found to stimulate $CD8^+$ T cells. Although no experiments were carried out to understand the HLA restriction of this epitope, previous work identified this epitope as restricted to HLA-B*07:02[299], an allele carried by participant 012c3k1. Restriction mapping experiments involve the use of a library of antigen presenting cells (APC) with known HLA alleles to be used to stimulate the T cells. At least one HLA molecule should be shared among the APC and the T cells. The identification of the HLA allele that binds to the peptide is determined by matching the allele that is shared

among the T cells and the APC which were able to promote the response. Although HLA alleles were determined[158], the lack of an APC library and cell availability did not allow the determination of HLA restriction.

The epitope NVKDTACLAKAYAQMWLL was also cross-reactive and it was also identified before vaccination. A similar epitope was identified in Zika virus as well as DENV infected individuals[290,296] indicating the possibility that this response may have been raised as a result of a previous DENV infection, suggested by the presence of NAb specific to DENV1, 2 and 4 at baseline.

Participant 020C1K1 showed T cell responses to E (294-547) and NS5 (2526-2841) following JE vaccination. These epitopes were identified as GATWVDLVL and GAGHEEPMLMQSYGWNLV for E and NS5 respectively. Interestingly, the response to GATWVDLVL was cross-reactive with DENV1/3 and 2 and was present before vaccination; whereas the response to GAGHEEPMLMQSYGWNLV not only was not cross-reactive but was not detected at baseline. This indicated that the presence of pre-existing responses (albeit low level) from previous flavivirus infection did not inhibit *de novo* responses specific to the JE vaccine. Because SA14-14-2 is replication competent, this can be inferred to mean that previous responses also do not inhibit new responses to a newly infecting virus.

The epitope GAGHEEPMLMQSYGWNLV was also similar to a previously identified CD8 T cell responses to JEV; however cross-reactivity against DENV was not tested[157].

The response to GATWVDLVL peptide was also described in a previous work in a cohort of patients with WNV infection[300].

The ALRGLPVRY peptide was initially identified by Dr Turtle on 001c1k1 and cross-reactive responses to DENV and JE vaccine peptides were observed. Interestingly, the response against the vaccine variant was less efficient, likely indicating priming with a different virus. Indeed, the response was present before vaccination possibly raised following a DENV2 exposure as the subject was showing the highest NAb titre against this serotype. My experiment on this epitope confirmed the cross-reactive nature of this epitope also against DENV1 and 3 and the less efficiency cross-reactive response of the vaccine variant. This epitope was also previously characterized by Zivny and colleagues[301]. They described a highly cross-reactive response measured by lysis of peptide pulsed APC among DENV2 and DENV3 variants. This is in agreement with the findings described here, although the priming virus was different in our study (DENV2 as opposed to yellow fever and DENV3 vaccine).

T cell cross-reactivity among different flaviviruses has been previously studied. Recently, strongly cross-reactive T cell responses were found in healthy JEV exposed volunteers but not in JE patients[157]. With the recent Zika outbreak, cross-reactivity among Zika and DENV has been an important topic. T cell epitopes raised following DENV infection or a tetravalent dengue vaccine were found to cross-react with Zika variants[255].

Although cross-reactive T cells have been described, their role in protection is not fully understood. Initial reports described a skewed T cell response upon stimulation with cross-reactive peptides with an imbalance in TNFα production[206]. Recently however, more evidence suggests a possible protective role for cross-reactive T cells[157]. Moreover, it was observed that T cells primed by DENV2 infection were involved in viral clearance and protection of mice from Zika virus challenge[254].

In summary, T cell responses were observed following LAJV in participants who developed a NAb response and in those who did not. Together with *de novo* responses (cross-reactive and non cross-reactive), boosted cross-reactive memory responses were also observed indicating that previous flavivirus infection did not inhibit new T cell priming.

A limitation of the project was the small sample size and the limited number of cells available for some participants. Finally, the peptide libraries of the 5 viruses used did not have fully matching peptides, therefore differences of responses among each virus variant may be due to variation in epitope location within the library peptide. HLA restriction and fine mapping experiments using tetramers would have helped to understand if OAS at T cell level occurs following LAJV in DENV exposed individuals. However, HLA restriction mapping would have required to build a panel of lymphoblastoid cell lines which was not possible to achieve during the limited period in India.

Conclusion

The present study aimed to examine the antibody and T cell response to the live attenuated Japanese encephalitis vaccine (LAJV) in healthy adult flavivirus exposed individuals. The study was conducted in India, a country where both Japanese encephalitis virus (JEV) and dengue virus (DENV), two flaviviruses, circulate. Samples collected before vaccination were crucial for having a clear understanding of the response to the vaccine as most of the vaccinees were DENV seropositive at baseline.

The data obtained in this study are consistent with the low immunogenicity of LAJV in adults in India already published in other studies. Using a stringent measure of the $PRNT_{70}$ value to analyse the NAb response to the LAJV, only three out of 16 (18.75%) of the volunteers responded. Additionally, the volunteers who responded to the vaccine showed low NAb titres specific to JEV which peaked at 4 weeks and started to wane by week 8 following vaccination.

This study described for the first time the phenomenon of original antigenic sin (OAS) among flaviviruses of different serogroups. OAS was observed following LAJV in a total of 3 volunteers who showed an increase of the NAb titre to DENV rather than to JEV. Among the DENV seropositive individuals, OAS was observed in approximately 30% of vaccinees. OAS was observed among responders and non-responders, and I was not able to determine a relationship between OAS and decreased immunogenicity of the vaccine in the population studied, due to the small numbers involved in this study. On contrary, data suggested that previous DENV exposure increases immunogenicity of the LAJV.

Previous work published by our group showed that the LAJV was able to induce T cell responses against all JEV proteins which peaked at 2 weeks following vaccination. Interestingly, T cell responses specific to JEV were detected following vaccination even in volunteers without NAb response. In this project I demonstrated that the vaccine was able to induce *de novo* DENV cross-reactive as well as JEV specific T cell responses. Finally, previous cross-reactive DENV responses did not inhibit *de novo* T cell responses.

Although it was not possible to show that OAS accounts for the low immunogenicity of the vaccine, this phenomenon remains a plausible explanation which needs further investigation. Such studies could also confirm whether previous DENV infection may enhance the immunogenicity of the vaccine in some people. It remains possible that previous DENV infection could have opposite effects on LAJV immunogenicity in different people.

Understanding the reasons for this, and whether this translates into differences in clinical protection, are crucial outstanding questions.

To have a better understanding of the antibody response, serum samples collected before and after vaccination could be analysed by ELISA using whole virion and NS1 protein of JEV and DENV1-4 as antigen. This assay could reveal more subjects responding to the vaccine by producing non-neutralising antibodies and the full degree of cross-reactivity induced by the vaccine. Another aspect of the antibody response not addressed here but that merits further investigation is the phenomenon of antibody dependent enhancement (ADE). Serial dilutions of any serum sample containing antibody against DENV shows ADE *in vitro*. As antibody titres specific for DENV change following LAJV administration, it would be interesting to determine if ADE occurs at different dilutions before and after vaccination, and the balance of neutralising versus enhancing antibody at different time points. Future work could also aim to definitively show the phenomenon of OAS for T cells following vaccination with LAJV in volunteers with previous DENV exposure. This could be achieved by first identifying minimal HLA restricted T cell epitopes of DENV in DENV exposed, JEV seronegative subjects. In order to show OAS, it would be necessary to identify epitopes that vary between DENV and JEV, and are partially cross-reactive before JE vaccination. The specificity of the T cells would then be studied before and after vaccination by using differentially labelled HLA class I-peptide tetramers loaded with the DENV peptide and the corresponding JEV variant. OAS would be demonstrated if the T cell response to the DENV epitope increased in magnitude more than the JEV peptide after JE vaccination, or if the antigen specific T cell population appearing after vaccination bound the original DENV serotype better than the JEV epitope. Finally, it will be interesting to determine if administration of a second dose of the LAJV would improve the immunogenicity of the vaccine in adult Indian population.

References

1. Grossman, R. A., Edelman, R., Willhight, M., Pantuwatana, S. & Udomsakdi, S. Study of Japanese encephalitis virus in Chiangmai Valley, Thailand. 3. Human seroepidemiology and inapparent infections. *Am. J. Epidemiol.* **98**, 133–149 (1973).

2. Southam, C. M. Serological studies of encephalitis in Japan. II. Inapparent infections by Japanese B encephalitis virus. *J. Infect. Dis.* **99**, 163–169 (1956).

3. Vaughn, D. W. & Hoke, C. H. The Epidemiology of Japanese Encephalitis: Prospects for Prevention. *Epidemiol. Rev.* **14**, 197–221 (1992).

4. Rudolph, K. E., Moloney, R. M., Kmush, B., Cummings, D. A. T. & Lessler, J. Incubation Periods of Mosquito-Borne Viral Infections: A Systematic Review. *Am. J. Trop. Med. Hyg.* **90**, 882–891 (2014).

5. Turtle, L. & Solomon, T. *Japanese encephalitis virus infection*. *Birkhauser Advances in Infectious Diseases* **123**, (Elsevier B.V., 2013).

6. Solomon, T. *et al.* Japanese encephalitis. *J. Neurol. Neurosurg. Psychiatry* **68**, 405–415 (2000).

7. Solomon, T. *et al.* Seizures and raised intracranial pressure in Vietnamese patients with Japanese encephalitis. *Brain* **125**, 1084–1093 (2002).

8. Solomon, T. *et al.* Poliomyelitis-like illness due to Japanese encephalitis virus. *Lancet* **351**, 1094–1097 (1998).

9. Misra, U. K., Kalita, J., Jain, S. K. & Mathur, a. Radiological and neurophysiological changes in Japanese encephalitis. *J. Neurol. Neurosurg. Psychiatry* **57**, 1484–1487 (1994).

10. Turtle, L. & Solomon, T. Japanese encephalitis — the prospects for new treatments. *Nat. Rev. Neurol.* **14**, 298–313 (2018).

11. Burrell, C. J. *et al.* Flaviviruses. in *Fenner and White's Medical Virology* 493–518 (Elsevier, 2017).

12. Manokaran, G. *et al.* Dengue subgenomic RNA binds TRIM25 to inhibit interferon expression for epidemiological fitness. *Science* **350**, 217–221 (2015).

13. Mori, Y. *et al.* Nuclear localization of Japanese encephalitis virus core protein enhances viral replication. *J. Virol.* **79**, 3448–3458 (2005).

14. Konishi, E. & Mason, P. W. Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. *J. Virol.* **67**, 1672–1675 (1993).

15. Lorenz, I. C., Allison, S. L., Heinz, F. X. & Helenius, A. Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. *J. Virol.* **76**, 5480–5491 (2002).

16. Mukhopadhyay, S., Kuhn, R. J. & Rossmann, M. G. A structural perspective of the flavivirus life cycle. *Nat. Rev. Microbiol.* **3**, 13–22 (2005).

17. Zhang, X. *et al.* Structure of acidic pH dengue virus showing the fusogenic

glycoprotein trimers. *J. Virol.* **89**, 743–750 (2015).

18. Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. Structure of the dengue virus envelope protein after membrane fusion. *Nature* **427**, 313–319 (2004).

19. Heinz, F. X. & Stiasny, K. The Antigenic Structure of Zika Virus and Its Relation to Other Flaviviruses: Implications for Infection and Immunoprophylaxis. *Microbiol. Mol. Biol. Rev.* **81**, 1–27 (2017).

20. Rastogi, M., Sharma, N. & Singh, S. K. Flavivirus NS1: a multifaceted enigmatic viral protein. *Virol. J.* **13**, 131 (2016).

21. Melian, E. B. *et al.* NS1' of flaviviruses in the Japanese encephalitis virus serogroup is a product of ribosomal frameshifting and plays a role in viral neuroinvasiveness. *J. Virol.* **84**, 1641–1647 (2010).

22. Rossi, S. L., Fayzulin, R., Dewsbury, N., Bourne, N. & Mason, P. W. Mutations in West Nile virus nonstructural proteins that facilitate replicon persistence in vitro attenuate virus replication in vitro and in vivo. *Virology* **364**, 184–195 (2007).

23. Mackenzie, J. M., Khromykh, A. A., Jones, M. K. & Westaway, E. G. Subcellular Localization and Some Biochemical Properties of the Flavivirus Kunjin Nonstructural Proteins NS2A and NS4A. *Virology* **245**, 203–215 (1998).

24. Liu, W. J., Chen, H. B. & Khromykh, A. A. Molecular and functional analyses of Kunjin virus infectious cDNA clones demonstrate the essential roles for NS2A in virus assembly and for a nonconservative residue in NS3 in RNA replication. *J. Virol.* **77**, 7804–7813 (2003).

25. Xie, X., Gayen, S., Kang, C., Yuan, Z. & Shi, P.-Y. Membrane topology and function of dengue virus NS2A protein. *J. Virol.* **87**, 4609–4622 (2013).

26. Tu, Y.-C. *et al.* Blocking double-stranded RNA-activated protein kinase PKR by Japanese encephalitis virus nonstructural protein 2A. *J. Virol.* **86**, 10347–10358 (2012).

27. Liu, W. J. *et al.* A Single Amino Acid Substitution in the West Nile Virus Nonstructural Protein NS2A Disables Its Ability To Inhibit Alpha/Beta Interferon Induction and Attenuates Virus Virulence in Mice. *J. Virol.* **80**, 2396–2404 (2006).

28. Gupta, G., Lim, L. & Song, J. NMR and MD Studies Reveal That the Isolated Dengue NS3 Protease Is an Intrinsically Disordered Chymotrypsin Fold Which Absolutely Requests NS2B for Correct Folding and Functional Dynamics. *PLoS One* **10**, e0134823 (2015).

29. Li, X.-D. *et al.* Transmembrane Domains of NS2B Contribute to both Viral RNA Replication and Particle Formation in Japanese Encephalitis Virus. *J. Virol.* **90**, 5735–5749 (2016).

30. Ma, L. *et al.* Host factor SPCS1 regulates the replication of Japanese encephalitis virus through interactions with transmembrane domains of NS2B. *J. Virol.* **92**, 197–218 (2018).

31. Luo, D. *et al.* Crystal structure of the NS3 protease-helicase from dengue virus. *J. Virol.* **82**, 173–183 (2008).

32. Yamashita, T. *et al.* Crystal structure of the catalytic domain of Japanese encephalitis virus NS3 helicase/nucleoside triphosphatase at a resolution of 1.8 Å. *Virology* **373**, 426–436 (2008).

33. Aguirre, S. *et al.* DENV Inhibits Type I IFN Production in Infected Cells by Cleaving Human STING. *PLoS Pathog.* **8**, e1002934 (2012).

34. Barber, G. N. STING-dependent signaling. *Nat. Immunol.* **12**, 929–930 (2011).

35. Chan, Y. K. & Gack, M. U. A phosphomimetic-based mechanism of dengue virus to antagonize innate immunity. *Nat. Immunol.* **17**, 523–530 (2016).

36. Wang, C.-C., Huang, Z.-S., Chiang, P.-L., Chen, C.-T. & Wu, H.-N. Analysis of the nucleoside triphosphatase, RNA triphosphatase, and unwinding activities of the helicase domain of dengue virus NS3 protein. *FEBS Lett.* **583**, 691–696 (2009).

37. Gebhard, L. G., Kaufman, S. B. & Gamarnik, A. V. Novel ATP-Independent RNA Annealing Activity of the Dengue Virus NS3 Helicase. *PLoS One* **7**, e36244 (2012).

38. Decroly, E., Ferron, F., Lescar, J. & Canard, B. Conventional and unconventional mechanisms for capping viral mRNA. *Nat. Rev. Microbiol.* **10**, 51–65 (2012).

39. Lin, C., Amberg, S. M., Chambers, T. J. & Rice, C. M. Cleavage at a novel site in the NS4A region by the yellow fever virus NS2B-3 proteinase is a prerequisite for processing at the downstream 4A/4B signalase site. *J. Virol.* **67**, 2327–2335 (1993).

40. Ambrose, R. L. & Mackenzie, J. M. West Nile Virus Differentially Modulates the Unfolded Protein Response To Facilitate Replication and Immune Evasion. *J. Virol.* **85**, 2723–2732 (2011).

41. McLean, J. E., Wudzinska, A., Datan, E., Quaglino, D. & Zakeri, Z. Flavivirus NS4A-induced autophagy protects cells against death and enhances virus replication. *J. Biol. Chem.* **286**, 22147–22159 (2011).

42. Chatel-Chaix, L. *et al.* Dengue Virus Perturbs Mitochondrial Morphodynamics to Dampen Innate Immune Responses. *Cell Host Microbe* **20**, 342–356 (2016).

43. Courtney, S. C., Scherbik, S. V., Stockman, B. M. & Brinton, M. A. West Nile Virus Infections Suppress Early Viral RNA Synthesis and Avoid Inducing the Cell Stress Granule Response. *J. Virol.* **86**, 3647–3657 (2012).

44. Lu, G. & Gong, P. Crystal Structure of the Full-Length Japanese Encephalitis Virus NS5 Reveals a Conserved Methyltransferase-Polymerase Interface. *PLoS Pathog.* **9**, e1003549 (2013).

45. Lin, R.-J., Chang, B.-L., Yu, H.-P., Liao, C.-L. & Lin, Y.-L. Blocking of Interferon-Induced Jak-Stat Signaling by Japanese Encephalitis Virus NS5 through a Protein Tyrosine Phosphatase-Mediated Mechanism. *J. Virol.* **80**, 5908–5918 (2006).

46. Yang, T.-C. *et al.* Proteomic analysis for Type I interferon antagonism of Japanese encephalitis virus NS5 protein. *Proteomics* **13**, 3442–3456 (2013).

47. Ye, J. *et al.* Japanese Encephalitis Virus NS5 Inhibits Type I Interferon (IFN) Production by Blocking the Nuclear Translocation of IFN Regulatory Factor 3 and NF-κB. *J. Virol.* **91**, 39–47 (2017).

48. Lubick, K. J. *et al.* Flavivirus Antagonism of Type I Interferon Signaling Reveals

Prolidase as a Regulator of IFNAR1 Surface Expression. *Cell Host Microbe* **18**, 61–74 (2015).

49. Evans, J. D., Crown, R. A., Sohn, J. A. & Seeger, C. West Nile Virus Infection Induces Depletion of IFNAR1 Protein Levels. *Viral Immunol.* **24**, 253–263 (2011).

50. Mazzon, M., Jones, M., Davidson, A., Chain, B. & Jacobs, M. Dengue Virus NS5 Inhibits Interferon-α Signaling by Blocking Signal Transducer and Activator of Transcription 2 Phosphorylation. *J. Infect. Dis.* **200**, 1261–1270 (2009).

51. Ashour, J., Laurent-Rolle, M., Shi, P.-Y. & Garcia-Sastre, A. NS5 of Dengue Virus Mediates STAT2 Binding and Degradation. *J. Virol.* **83**, 5408–5418 (2009).

52. Grant, A. *et al.* Zika Virus Targets Human STAT2 to Inhibit Type I Interferon Signaling. *Cell Host Microbe* **19**, 882–890 (2016).

53. Chen, Y. C., Wang, S. Y. & King, C. C. Bacterial lipopolysaccharide inhibits dengue virus infection of primary human monocytes/macrophages by blockade of virus entry via a CD14-dependent mechanism. *J. Virol.* **73**, 2650–2657 (1999).

54. Miller, J. L. *et al.* The mannose receptor mediates dengue virus infection of macrophages. *PLoS Pathog.* **4**, e17 (2008).

55. Tassaneetrithep, B. *et al.* DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J. Exp. Med.* **197**, 823–829 (2003).

56. Hung, C.-Y., Tsai, M.-C., Wu, Y.-P. & Wang, R. Y. L. Identification of heat-shock protein 90 beta in Japanese encephalitis virus-induced secretion proteins. *J. Gen. Virol.* **92**, 2803–2809 (2011).

57. Nain, M. *et al.* GRP78 Is an Important Host Factor for Japanese Encephalitis Virus Entry and Replication in Mammalian Cells. *J. Virol.* **91**, 2274–2316 (2017).

58. Das, S., Laxminarayana, S. V., Chandra, N., Ravi, V. & Desai, A. Heat shock protein 70 on Neuro2a cells is a putative receptor for Japanese encephalitis virus. *Virology* **385**, 47–57 (2009).

59. van der Schaar, H. M. *et al.* Dissecting the Cell Entry Pathway of Dengue Virus by Single-Particle Tracking in Living Cells. *PLoS Pathog.* **4**, e1000244 (2008).

60. Rey, F. A., Stiasny, K., Vaney, M., Dellarole, M. & Heinz, F. X. The bright and the dark side of human antibody responses to flaviviruses: lessons for vaccine design. *EMBO Rep.* **19**, 206–224 (2018).

61. Li, L. *et al.* The flavivirus precursor membrane-envelope protein complex: structure and maturation. *Science* **319**, 1830–1834 (2008).

62. Zheng, A., Yuan, F., Kleinfelter, L. M. & Kielian, M. A toggle switch controls the low pH-triggered rearrangement and maturation of the dengue virus envelope proteins. *Nat. Commun.* **5**, 3877 (2014).

63. Yu, I.-M. *et al.* Structure of the immature dengue virus at low pH primes proteolytic maturation. *Science* **319**, 1834–1837 (2008).

64. Guirakhoo, F., Heinz, F. X., Mandl, C. W., Holzmann, H. & Kunz, C. Fusion activity of flaviviruses: comparison of mature and immature (prM-containing) tick-borne encephalitis virions. *J. Gen. Virol.* **72**, 1323–1329 (1991).

65. Zhang, Y. *et al.* Structures of immature flavivirus particles. *EMBO J.* **22**, 2604–2613 (2003).

66. Solomon, T. Control of Japanese encephalitis--within our grasp? *N. Engl. J. Med.* **355**, 869–871 (2006).

67. Lord, J. S., Gurley, E. S. & Pulliam, J. R. C. Rethinking Japanese Encephalitis Virus Transmission: A Framework for Implicating Host and Vector Species. *PLoS Negl. Trop. Dis.* **9**, e0004074 (2015).

68. Quan, T. M., Thao, T. T. N., Duy, N. M., Nhat, T. M. & Clapham, H. Estimates of the global burden of Japanese encephalitis and the impact of vaccination from 2000-2015. *Elife* **9**, e51027 (2020).

69. Mogi, M. Relationship between number of human Japanese encephalitis cases and summer meteorological conditions in Nagasaki, Japan. *Am. J. Trop. Med. Hyg.* **32**, 170–174 (1983).

70. Hanna, J. N. *et al.* An outbreak of Japanese encephalitis in the Torres Strait, Australia, 1995. *Med. J. Aust.* **165**, 256–60 (1996).

71. Arai, S. *et al.* Japanese encephalitis: Surveillance and elimination effort in Japan from 1982 to 2004. *Jpn. J. Infect. Dis.* **61**, 333–338 (2008).

72. Lee, D. W. *et al.* Epidemiology of Japanese encephalitis in South Korea, 2007-2010. *Int. J. Infect. Dis.* **16**, e448–e452 (2012).

73. Wu, Y. C. *et al.* The epidemiology of Japanese encephalitis on Taiwan during 1966-1997. *Am. J. Trop. Med. Hyg.* **61**, 78–84 (1999).

74. Schuh, A. J., Ward, M. J., Leigh Brown, A. J. & Barrett, A. D. T. Dynamics of the emergence and establishment of a newly dominant genotype of Japanese encephalitis virus throughout Asia. *J. Virol.* **88**, 4522–4532 (2014).

75. Gao, X. *et al.* Changing Geographic Distribution of Japanese Encephalitis Virus Genotypes, 1935–2017. *Vector-Borne Zoonotic Dis.* **19**, 35–44 (2019).

76. Kabilan, L. *et al.* Japanese encephalitis in India: an overview. *Indian J. Pediatr.* **71**, 609–615 (2004).

77. Kumari, R. & Joshi, P. A review of Japanese encephalitis in Uttar Pradesh, India. *WHO South-East Asia J. Public Heal.* **1**, 1–374 (2012).

78. Borthakur, A., Das, N. & Bora, B. Data from the World Health Organization (WHO) National Network Laboratory for Japanese Encephalitis. *J. Glob. Infect. Dis.* **5**, 76–9 (2013).

79. YK, G. *et al.* A large outbreak of Japanese encephalitis predominantly among adults in northern region of West Bengal, India. *J. Med. Virol.* **88**, 2004–11 (2016).

80. Medhi, M. *et al.* Incidence of Japanese Encephalitis amongst acute encephalitis syndrome cases in upper Assam districts from 2012 to 2014: A report from a tertiary care hospital. *Indian J. Med. Res.* **146**, 267–271 (2017).

81. Singh, A. *et al.* A Japanese Encephalitis Vaccine From India Induces Durable and Cross-protective Immunity Against Temporally and Spatially Wide-ranging Global Field Strains. *J. Infect. Dis.* **212**, 715–725 (2015).

82. Muniaraj, M. & Rajamannar, V. Impact of SA 14-14-2 vaccination on the occurrence of Japanese encephalitis in India. *Hum. Vaccin. Immunother.* **15**, 834–840 (2019).

83. Hoke, C. H. *et al.* Protection against Japanese Encephalitis by Inactivated Vaccines. *N. Engl. J. Med.* **319**, 608–614 (1988).

84. Poland, J. D., Bruce Cropp, C., Craven, R. B. & Monath, T. P. Evaluation of the Potency and Safety of Inactivated Japanese Encephalitis Vaccine in US Inhabitants. *J. Infect. Dis.* **161**, 878–882 (1990).

85. Halstead, S. B. & Thomas, S. J. New Vaccines for Japanese Encephalitis. *Curr. Infect. Dis. Rep.* **12**, 174–180 (2010).

86. Okada, K. *et al.* Safety and immunogenicity of a freeze-dried, cell culture-derived Japanese encephalitis vaccine (Inactivated) (JEBIK(®)V) in children. *Vaccine* **30**, 5967–5972 (2012).

87. Yun, K. W. *et al.* Safety and immunogenicity of a freeze-dried, Vero cell culture-derived, inactivated Japanese encephalitis vaccine (KD-287, ENCEVAC®) versus a mouse brain-derived inactivated Japanese encephalitis vaccine in children: a phase III, multicenter, double-blinded, randomized trial. *BMC Infect. Dis.* **15**, 7 (2015).

88. Chanthavanich, P. *et al.* Immunogenicity and safety of inactivated chromatographically purified Vero cell-derived Japanese encephalitis vaccine in Thai children. *Hum. Vaccin. Immunother.* **14**, 900–905 (2018).

89. Cramer, J. P. *et al.* Immunogenicity and safety of the inactivated Japanese encephalitis vaccine IXIARO® in elderly subjects: Open-label, uncontrolled, multi-center, phase 4 study. *Vaccine* **34**, 4579–4585 (2016).

90. Dubischar, K. L. *et al.* Immunogenicity of the Inactivated Japanese Encephalitis Virus Vaccine IXIARO in Children From a Japanese Encephalitis Virus-endemic Region. *Pediatr. Infect. Dis. J.* **36**, 898–904 (2017).

91. Dubischar, K. L. *et al.* Safety of the Inactivated Japanese Encephalitis Virus Vaccine IXIARO in Children. *Pediatr. Infect. Dis. J.* **36**, 889–897 (2017).

92. Jelinek, T. *et al.* Safety and immunogenicity of an inactivated Vero cell_derived Japanese encephalitis vaccine (IXIARO®, JESPECT®) in a pediatric population in JE non-endemic countries: An uncontrolled, open-label phase 3 study. *Travel Med. Infect. Dis.* **22**, 18–24 (2018).

93. Yu, Y. Phenotypic and genotypic characteristics of Japanese encephalitis attenuated live vaccine virus SA14-14-2 and their stabilities. *Vaccine* **28**, 3635–3641 (2010).

94. Yun, S.-I. *et al.* A Molecularly Cloned, Live-Attenuated Japanese Encephalitis Vaccine SA14-14-2 Virus: A Conserved Single Amino Acid in the ij Hairpin of the Viral E Glycoprotein Determines Neurovirulence in Mice. *PLoS Pathog.* **10**, e1004290 (2014).

95. Ye, Q. *et al.* A single nucleotide mutation in NS2A of Japanese encephalitis-live vaccine virus (SA14-14-2) ablates NS1' formation and contributes to attenuation. *J. Gen. Virol.* **93**, 1959–1964 (2012).

96. Zaman, K. *et al.* Lot-to-lot consistency of live attenuated SA 14-14-2 Japanese encephalitis vaccine manufactured in a good manufacturing practice facility and non-

inferiority with respect to an earlier product. *Vaccine* **32**, 6061–6066 (2014).

97. Feroldi, E. *et al.* Primary Immunization of Infants and Toddlers in Thailand with Japanese Encephalitis Chimeric Virus Vaccine in Comparison with SA14-14-2. *Pediatr. Infect. Dis. J.* **33**, 643–649 (2014).

98. Wijesinghe, P. R. *et al.* Safety and immunogenicity of live-attenuated Japanese encephalitis SA 14-14-2 vaccine co-administered with measles vaccine in 9-month-old infants in Sri Lanka. *Vaccine* **32**, 4751–4757 (2014).

99. Bista, M. B. *et al.* Efficacy of single-dose SA 14-14-2 vaccine against Japanese encephalitis: a case control study. *Lancet* **358**, 791–795 (2001).

100. Tandan, J. B. *et al.* Single dose of SA 14-14-2 vaccine provides long-term protection against Japanese encephalitis: A case–control study in Nepalese children 5 years after immunization. *Vaccine* **25**, 5041–5045 (2007).

101. Hennessy, S. *et al.* Effectiveness of live-attenuated Japanese encephalitis vaccine (SA14-14-2): a case-control study. *Lancet* **347**, 1583–1586 (1996).

102. Vashishtha, V. M. *et al.* Indian Academy of Pediatrics (IAP) recommended immunization schedule for children aged 0 through 18 years — India, 2013 and updates on immunization. *Indian Pediatr.* **50**, 1095–1108 (2013).

103. Tandale, B. V *et al.* Effectiveness of Japanese encephalitis SA 14-14-2 live attenuated vaccine among Indian children: Retrospective 1:4 matched case-control study. *J. Infect. Public Health* **11**, 713–719 (2018).

104. Torresi, J., McCarthy, K., Feroldi, E. & Méric, C. Immunogenicity, safety and tolerability in adults of a new single-dose, live-attenuated vaccine against Japanese encephalitis: Randomised controlled phase 3 trials. *Vaccine* **28**, 7993–8000 (2010).

105. Monath, T. P. *et al.* Chimeric Live, Attenuated Vaccine against Japanese Encephalitis (ChimeriVax-JE): Phase 2 Clinical Trials for Safety and Immunogenicity, Effect of Vaccine Dose and Schedule, and Memory Response to Challenge with Inactivated Japanese Encephalitis Antigen. *J. Infect. Dis.* **188**, 1213–1230 (2003).

106. Monath, T. P. *et al.* Clinical proof of principle for ChimeriVax^{TM}: recombinant live, attenuated vaccines against flavivirus infections. *Vaccine* **20**, 1004–1018 (2002).

107. Capeding, M. R. *et al.* Five-Year Antibody Persistence Following a Japanese Encephalitis Chimeric Virus Vaccine (JE-CV) Booster in JE-CV-Primed Children in the Philippines. *J. Infect. Dis.* **217**, 567–571 (2018).

108. Kosalaraksa, P. *et al.* Long-term Immunogenicity of a Single Dose of Japanese Encephalitis Chimeric Virus Vaccine in Toddlers and Booster Response 5 Years After Primary Immunization. *Pediatr. Infect. Dis. J.* **36**, e108–e113 (2017).

109. Vu, T. D. *et al.* Immunogenicity and safety of a single dose of a live attenuated Japanese encephalitis chimeric virus vaccine in Vietnam: A single-arm, single-center study. *Int. J. Infect. Dis.* **66**, 137–142 (2018).

110. Thompson, M. R., Kaminski, J. J., Kurt-Jones, E. A. & Fitzgerald, K. A. Pattern recognition receptors and the innate immune response to viral infection. *Viruses* **3**, 920–940 (2011).

111. Wieland, S. F. *et al.* Searching for interferon-induced genes that inhibit hepatitis B virus replication in transgenic mouse hepatocytes. *J. Virol.* **77**, 1227–1236 (2003).

112. Yang, P. *et al.* The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway. *Nat. Immunol.* **11**, 487–494 (2010).

113. Rathinam, V. A. K. *et al.* The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat. Immunol.* **11**, 395–402 (2010).

114. Murphy, K. *Janeway'S Immunobiology, 7Th Edition*. *Garland Science* **USA**, (2012).

115. Tokuyasu, T. A. & Huang, J.-D. A primer on recent developments in cancer immunotherapy, with a focus on neoantigen vaccines. *J. Cancer Metastasis Treat.* **4**, 2 (2018).

116. Schmid, M. A. & Harris, E. Monocyte recruitment to the dermis and differentiation to dendritic cells increases the targets for dengue virus replication. *PLoS Pathog.* **10**, e1004541 (2014).

117. Cao, S. *et al.* Japanese encephalitis Virus wild strain infection suppresses dendritic cells maturation and function, and causes the expansion of regulatory T cells. *Virol. J.* **8**, 39 (2011).

118. Aoki, K. *et al.* Type-I interferon response affects an inoculation dose-independent mortality in mice following Japanese encephalitis virus infection. *Virol. J.* **11**, 105 (2014).

119. Han, Y. W. *et al.* Distinct Dictation of Japanese Encephalitis Virus-Induced Neuroinflammation and Lethality via Triggering TLR3 and TLR4 Signal Pathways. *PLoS Pathog.* **10**, e1004319 (2014).

120. Solomon, T. *et al.* Interferon alfa-2a in Japanese encephalitis: a randomised double-blind placebo-controlled trial. *Lancet* **361**, 821–826 (2003).

121. German, A. C. *et al.* A preliminary neuropathological study of Japanese encephalitis in humans and a mouse model. *Trans. R. Soc. Trop. Med. Hyg.* **100**, 1135–1145 (2006).

122. Patabendige, A., Michael, B. D., Craig, A. G. & Solomon, T. Brain microvascular endothelial-astrocyte cell responses following Japanese encephalitis virus infection in an in vitro human blood-brain barrier model. *Mol. Cell. Neurosci.* **89**, 60–70 (2018).

123. Al-Obaidi, M. M. J. *et al.* Japanese encephalitis virus disrupts blood-brain barrier and modulates apoptosis proteins in THBMEC cells. *Virus Res.* **233**, 17–28 (2017).

124. Li, F. *et al.* Viral Infection of the Central Nervous System and Neuroinflammation Precede Blood-Brain Barrier Disruption during Japanese Encephalitis Virus Infection. *J. Virol.* **89**, 5602–5614 (2015).

125. Liou, M. L. & Hsu, C. Y. Japanese encephalitis virus is transported across the cerebral blood vessels by endocytosis in mouse brain. *Cell Tissue Res.* **293**, 389–394 (1998).

126. Johnson, R. T. *et al.* Japanese encephalitis: Immunocytochemical studies of viral antigen and Inflammatory cells in fatal cases. *Ann. Neurol.* **18**, 567–573 (1985).

127. Desai, A., Shankar, S. K., Ravi, V., Chandramuki, A. & Gourie-Devi, M. Japanese encephalitis virus antigen in the human brain and its topographic distribution. *Acta*

Neuropathol. **89**, 368–373 (1995).

128. Myint, K. S. A. *et al.* Neuropathogenesis of Japanese Encephalitis in a Primate Model. *PLoS Negl. Trop. Dis.* **8**, e2980 (2014).

129. Das, S., Mishra, M. K., Ghosh, J. & Basu, A. Japanese Encephalitis Virus infection induces IL-18 and IL-1β in microglia and astrocytes: Correlation with in vitro cytokine responsiveness of glial cells and subsequent neuronal death. *J. Neuroimmunol.* **195**, 60–72 (2008).

130. Nazmi, A., Dutta, K., Das, S. & Basu, A. Japanese Encephalitis Virus-Infected Macrophages Induce Neuronal Death. *J. Neuroimmune Pharmacol.* **6**, 420–433 (2011).

131. Konishi, E. *et al.* The anamnestic neutralizing antibody response is critical for protection of mice from challenge following vaccination with a plasmid encoding the Japanese encephalitis virus premembrane and envelope genes. *J. Virol.* **73**, 5527–5534 (1999).

132. Lubiniecki, A. S., Cypess, R. H. & Hammon, W. M. Passive immunity for arbovirus infection. II. Quantitative aspects of naturally and artificially acquired protection in mice for Japanese (B) encephalitis virus. *Am. J. Trop. Med. Hyg.* **22**, 535–542 (1973).

133. Fernandez, E. *et al.* Mouse and Human Monoclonal Antibodies Protect against Infection by Multiple Genotypes of Japanese Encephalitis Virus. *MBio* **9**, 8–18 (2018).

134. Kimura-Kuroda, J. & Yasui, K. Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies. *J. Immunol.* **141**, 3606–3610 (1988).

135. Oya, A. Japanese encephalitis vaccine. *Acta Paediatr. Jpn. Overseas Ed.* **30**, 175–184 (1988).

136. Hombach, J., Solomon, T., Kurane, I., Jacobson, J. & Wood, D. Report on a WHO consultation on immunological endpoints for evaluation of new Japanese encephalitis vaccines, WHO, Geneva, 2–3 September, 2004. *Vaccine* **23**, 5205–5211 (2005).

137. Beasley, D. W. . *et al.* Protection against Japanese encephalitis virus strains representing four genotypes by passive transfer of sera raised against ChimeriVax™-JE experimental vaccine. *Vaccine* **22**, 3722–3726 (2004).

138. Winter, P. M. *et al.* Proinflammatory Cytokines and Chemokines in Humans with Japanese Encephalitis. *J. Infect. Dis.* **190**, 1618–1626 (2004).

139. Libraty, D. H. *et al.* Clinical and immunological risk factors for severe disease in Japanese encephalitis. *Trans. R. Soc. Trop. Med. Hyg.* **96**, 173–178 (2002).

140. Lorsomrudee, W. *et al.* Fatal Outcome in Japanese Encephalitis. *Am. J. Trop. Med. Hyg.* **34**, 1203–1210 (1985).

141. Stettler, K. *et al.* Specificity, cross-reactivity, and function of antibodies elicited by Zika virus infection. *Science* **353**, 823–826 (2016).

142. Slon Campos, J. L., Mongkolsapaya, J. & Screaton, G. R. The immune response against flaviviruses. *Nat. Immunol.* **19**, 1189–1198 (2018).

143. Beltramello, M. *et al.* The Human Immune Response to Dengue Virus Is Dominated by Highly Cross-Reactive Antibodies Endowed with Neutralizing and Enhancing

Activity. *Cell Host Microbe* **8**, 271–283 (2010).

144. Aleyas, A. G. *et al.* Multifront Assault on Antigen Presentation by Japanese Encephalitis Virus Subverts CD8+ T Cell Responses. *J. Immunol.* **185**, 1429–1441 (2010).

145. Aleyas, A. G. *et al.* Impaired cross-presentation of CD8α $^{+}$ CD11c $^{+}$ dendritic cells by Japanese encephalitis virus in a TLR2/MyD88 signal pathway-dependent manner. *Eur. J. Immunol.* **42**, 2655–2666 (2012).

146. Aleyas, A. G. *et al.* Functional modulation of dendritic cells and macrophages by Japanese encephalitis virus through MyD88 adaptor molecule-dependent and -independent pathways. *J. Immunol.* **183**, 2462–2474 (2009).

147. Larena, M., Regner, M., Lee, E. & Lobigs, M. Pivotal role of antibody and subsidiary contribution of CD8+ T cells to recovery from infection in a murine model of Japanese encephalitis. *J. Virol.* **85**, 5446–5455 (2011).

148. Jain, N. *et al.* CD8 T cells protect adult naive mice from JEV-induced morbidity via lytic function. *PLoS Negl. Trop. Dis.* **11**, e0005329 (2017).

149. Murali-Krishna, K., Ravi, V. & Manjunath, R. Protection of adult but not newborn mice against lethal intracerebral challenge with Japanese encephalitis virus by adoptively transferred virus-specific cytotoxic T lymphocytes: requirement for L3T4+ T cells. *J. Gen. Virol.* **77**, 705–714 (1996).

150. Li, X.-F. *et al.* A chimeric dengue virus vaccine using Japanese encephalitis virus vaccine strain SA14-14-2 as backbone is immunogenic and protective against either parental virus in mice and nonhuman primates. *J. Virol.* **87**, 13694–13705 (2013).

151. Biswas, S. M. *et al.* Immunomodulatory cytokines determine the outcome of Japanese encephalitis virus infection in mice. *J. Med. Virol.* **82**, 304–310 (2010).

152. Biswas, S. M., Ayachit, V. M., Sapkal, G. N., Mahamuni, S. A. & Gore, M. M. Japanese encephalitis virus produces a CD4+ Th2 response and associated immunoprotection in an adoptive-transfer murine model. *J. Gen. Virol.* **90**, 818–826 (2009).

153. Saxena, V., Mathur, A., Krishnani, N. & Dhole, T. N. An insufficient anti-inflammatory cytokine response in mouse brain is associated with increased tissue pathology and viral load during Japanese encephalitis virus infection. *Arch. Virol.* **153**, 283–292 (2008).

154. Kumar, P., Sulochana, P., Nirmala, G., Haridattatreya, M. & Satchidanandam, V. Conserved amino acids 193-324 of non-structural protein 3 are a dominant source of peptide determinants for CD4+ and CD8+ T cells in a healthy Japanese encephalitis virus-endemic cohort. *J. Gen. Virol.* **85**, 1131–1143 (2004).

155. Kumar, P. *et al.* Cell-mediated immune responses in healthy children with a history of subclinical infection with Japanese encephalitis virus: analysis of CD4+ and CD8+ T cell target specificities by intracellular delivery of viral proteins using the human immunodeficiency virus Tat protein transduction domain. *J. Gen. Virol.* **85**, 471–482 (2004).

156. Kumar, P. *et al.* Impaired T helper 1 function of nonstructural protein 3-specific T cells in Japanese patients with encephalitis with neurological sequelae. *J. Infect. Dis.*

189, 880–891 (2004).

157. Turtle, L. *et al.* Human T cell responses to Japanese encephalitis virus in health and disease. *J. Exp. Med.* **213**, 1331–1352 (2016).

158. Turtle, L. *et al.* Cellular Immune Responses to Live Attenuated Japanese Encephalitis (JE) Vaccine SA14-14-2 in Adults in a JE/Dengue Co-Endemic Area. *PLoS Negl. Trop. Dis.* **11**, e0005263 (2017).

159. Guzman, M. G. *et al.* Dengue: a continuing global threat. *Nat. Rev. Microbiol.* **8**, 7–16 (2010).

160. Bhatt, S. *et al.* The global distribution and burden of dengue. *Nature* **496**, 504–507 (2013).

161. Duong, V. *et al.* Asymptomatic humans transmit dengue virus to mosquitoes. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 14688–14693 (2015).

162. The World Health Organization (WHO). *Dengue Guidelines for diagnosis, treatment, prevention and control*. **France**, (WHO Library, 2009).

163. Prasert T. *Monograph on dengue/dengue haemorrhagic fever.* (World Health Organization, Regional Office for South-East Asia, 1996).

164. Guzmán, M. G. *et al.* Epidemiologic studies on Dengue in Santiago de Cuba, 1997. *Am. J. Epidemiol.* **152**, 793–799 (2000).

165. Halstead, S. B. Observations related to pathogensis of dengue hemorrhagic fever. VI. Hypotheses and discussion. *Yale J. Biol. Med.* **42**, 350–362 (1970).

166. Hammond, S. N. *et al.* Differences in dengue severity in infants, children and adults in a 3-year hospital-based study in Nicaragua. *Am. J. Trop. Med. Hyg.* **73**, 1063–1070 (2005).

167. Screaton, G., Mongkolsapaya, J., Yacoub, S. & Roberts, C. New insights into the immunopathology and control of dengue virus infection. *Nat. Rev. Immunol.* **15**, 745–759 (2015).

168. Mathew, A. *et al.* B-Cell Responses During Primary and Secondary Dengue Virus Infections in Humans. *J. Infect. Dis.* **204**, 1514–1522 (2011).

169. de Alwis, R. *et al.* In-Depth Analysis of the Antibody Response of Individuals Exposed to Primary Dengue Virus Infection. *PLoS Negl. Trop. Dis.* **5**, e1188 (2011).

170. Waggoner, J. J. *et al.* Homotypic Dengue Virus Reinfections in Nicaraguan Children. *J. Infect. Dis.* **214**, 986–993 (2016).

171. Sabin, A. B. Research on dengue during World War II. *Am. J. Trop. Med. Hyg.* **1**, 30–50 (1952).

172. Patel, B. *et al.* Dissecting the human serum antibody response to secondary dengue virus infections. *PLoS Negl. Trop. Dis.* **11**, e0005554 (2017).

173. Priyamvada, L. *et al.* B Cell Responses during Secondary Dengue Virus Infection Are Dominated by Highly Cross-Reactive, Memory-Derived Plasmablasts. *J. Virol.* **90**, 5574–5585 (2016).

174. Halstead, S. B. & O'Rourke, E. J. Dengue viruses and mononuclear phagocytes. I.

Infection enhancement by non-neutralizing antibody. *J. Exp. Med.* **146**, 201–217 (1977).

175. Halstead, S. B. & O'rourke, E. J. Antibody-enhanced dengue virus infection in primate leukocytes. *Nature* **265**, 739–741 (1977).

176. Katzelnick, L. C. *et al.* Antibody-dependent enhancement of severe dengue disease in humans. *Science* **358**, 929–932 (2017).

177. Chan, K. R. *et al.* Ligation of Fc gamma receptor IIB inhibits antibody-dependent enhancement of dengue virus infection. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 12479–12484 (2011).

178. Tsai, T.-T. *et al.* Antibody-Dependent Enhancement Infection Facilitates Dengue Virus-Regulated Signaling of IL-10 Production in Monocytes. *PLoS Negl. Trop. Dis.* **8**, e3320 (2014).

179. Ubol, S., Phuklia, W., Kalayanarooj, S. & Modhiran, N. Mechanisms of Immune Evasion Induced by a Complex of Dengue Virus and Preexisting Enhancing Antibodies. *J. Infect. Dis.* **201**, 923–935 (2010).

180. Kou, Z. *et al.* Human antibodies against dengue enhance dengue viral infectivity without suppressing type I interferon secretion in primary human monocytes. *Virology* **410**, 240–247 (2011).

181. Dejnirattisai, W. *et al.* Cross-reacting antibodies enhance dengue virus infection in humans. *Science* **328**, 745–748 (2010).

182. Rodenhuis-Zybert, I. A. *et al.* A fusion-loop antibody enhances the infectious properties of immature flavivirus particles. *J. Virol.* **85**, 11800–11808 (2011).

183. Dejnirattisai, W. *et al.* A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus. *Nat. Immunol.* **16**, 170–177 (2014).

184. Wahala, W. M. P. B., Kraus, A. A., Haymore, L. B., Accavitti-Loper, M. A. & de Silva, A. M. Dengue virus neutralization by human immune sera: Role of envelope protein domain III-reactive antibody. *Virology* **392**, 103–113 (2009).

185. Fibriansah, G. *et al.* A highly potent human antibody neutralizes dengue virus serotype 3 by binding across three surface proteins. *Nat. Commun.* **6**, 6341 (2015).

186. Fibriansah, G. *et al.* Cryo-EM structure of an antibody that neutralizes dengue virus type 2 by locking E protein dimers. *Science* **349**, 88–91 (2015).

187. Teoh, E. P. *et al.* The Structural Basis for Serotype-Specific Neutralization of Dengue Virus by a Human Antibody. *Sci. Transl. Med.* **4**, 139ra83-139ra83 (2012).

188. Fibriansah, G. *et al.* A potent anti-dengue human antibody preferentially recognizes the conformation of E protein monomers assembled on the virus surface. *EMBO Mol. Med.* **6**, 358–371 (2014).

189. Rouvinski, A. *et al.* Recognition determinants of broadly neutralizing human antibodies against dengue viruses. *Nature* **520**, 109–113 (2015).

190. Whitehead, S. S., Blaney, J. E., Durbin, A. P. & Murphy, B. R. Prospects for a dengue virus vaccine. *Nat. Rev. Microbiol.* **5**, 518–528 (2007).

191. Francis, T. On the Doctrine of Original Antigenic Sin. *Proceedings of the American Philosophical Society* **104**, 572–578

192. Davenport, F. M. & Hennessy, A. V. A serologic recapitulation of past experiences with influenza A; antibody response to monovalent vaccine. *J. Exp. Med.* **104**, 85–97 (1956).

193. Kim, J. H., Skountzou, I., Compans, R. & Jacob, J. Original Antigenic Sin Responses to Influenza Viruses. *J. Immunol.* **183**, 3294–3301 (2009).

194. Linderman, S. L. & Hensley, S. E. Antibodies with 'Original Antigenic Sin' Properties Are Valuable Components of Secondary Immune Responses to Influenza Viruses. *PLoS Pathog.* **12**, e1005806 (2016).

195. Halstead, S. B., Rojanasuphot, S. & Sangkawibha, N. Original antigenic sin in dengue. *Am. J. Trop. Med. Hyg.* **32**, 154–156 (1983).

196. Berry, J. D., Peeling, R. W. & Brunham, R. C. Analysis of the original antigenic sin antibody response to the major outer membrane protein of Chlamydia trachomatis. *J. Infect. Dis.* **179**, 180–186 (1999).

197. Klenerman, P. & Zinkernagel, R. M. Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes. *Nature* **394**, 482–485 (1998).

198. Ndifon, W. A simple mechanistic explanation for original antigenic sin and its alleviation by adjuvants. *J. R. Soc. Interface* **12**, 1–8 (2015).

199. Kim, J. H., Davis, W. G., Sambhara, S. & Jacob, J. Strategies to alleviate original antigenic sin responses to influenza viruses. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 13751–13756 (2012).

200. Liu, X. S. *et al.* Overcoming original antigenic sin to generate new CD8 T cell IFN-gamma responses in an antigen-experienced host. *J. Immunol.* **177**, 2873–2879 (2006).

201. Chakradhar, S. Updated, augmented vaccines compete with original antigenic sin. *Nat. Med.* **21**, 540–541 (2015).

202. Mongkolsapaya, J. *et al.* Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat. Med.* **9**, 921–927 (2003).

203. Duangchinda, T. *et al.* Immunodominant T-cell responses to dengue virus NS3 are associated with DHF. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 16922–16927 (2010).

204. Appanna, R. *et al.* Cross-Reactive T-Cell Responses to the Nonstructural Regions of Dengue Viruses among Dengue Fever and Dengue Hemorrhagic Fever Patients in Malaysia. *Clin. Vaccine Immunol.* **14**, 969–977 (2007).

205. Kurane, I., Meager, A. & Ennis, F. A. Dengue virus-specific human T cell clones. Serotype crossreactive proliferation, interferon gamma production, and cytotoxic activity. *J. Exp. Med.* **170**, 763–775 (1989).

206. Mangada, M. M. & Rothman, A. L. Altered cytokine responses of dengue-specific CD4+ T cells to heterologous serotypes. *J. Immunol.* **175**, 2676–2683 (2005).

207. Bashyam, H. S., Green, S. & Rothman, A. L. Dengue virus-reactive CD8+ T cells display quantitative and qualitative differences in their response to variant epitopes of

heterologous viral serotypes. *J. Immunol.* **176**, 2817–2824 (2006).

208. Rothman, A. L. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. *Nat. Rev. Immunol.* **11**, 532–543 (2011).

209. Weiskopf, D. *et al.* Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8+ T cells. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 2046–2053 (2013).

210. Zompi, S. & Harris, E. Original antigenic sin in dengue revisited. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 8761–8762 (2013).

211. Hatch, S. *et al.* Intracellular Cytokine Production by Dengue Virus–specific T cells Correlates with Subclinical Secondary Infection. *J. Infect. Dis.* **203**, 1282–1291 (2011).

212. Yauch, L. E. *et al.* A Protective Role for Dengue Virus-Specific CD8+ T Cells. *J. Immunol.* **182**, 4865–4873 (2009).

213. Elong Ngono, A. *et al.* Protective Role of Cross-Reactive CD8 T Cells Against Dengue Virus Infection. *EBioMedicine* **13**, 284–293 (2016).

214. Dung, N. T. P. *et al.* Timing of CD8+ T Cell Responses in Relation to Commencement of Capillary Leakage in Children with Dengue. *J. Immunol.* **184**, 7281–7287 (2010).

215. St John, A. L. Influence of mast cells on dengue protective immunity and immune pathology. *PLoS Pathog.* **9**, e1003783 (2013).

216. Beatty, P. R. *et al.* Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination. *Sci. Transl. Med.* **7**, 304ra141-304ra141 (2015).

217. Modhiran, N. *et al.* Dengue virus NS1 protein activates cells via Toll-like receptor 4 and disrupts endothelial cell monolayer integrity. *Sci. Transl. Med.* **7**, 304ra142-304ra142 (2015).

218. Modhiran, N. *et al.* Dengue virus NS1 protein activates immune cells via TLR4 but not TLR2 or TLR6. *Immunol. Cell Biol.* **95**, 491–495 (2017).

219. Puerta-Guardo, H., Glasner, D. R. & Harris, E. Dengue Virus NS1 Disrupts the Endothelial Glycocalyx, Leading to Hyperpermeability. *PLOS Pathog.* **12**, e1005738 (2016).

220. Glasner, D. R. *et al.* Dengue virus NS1 cytokine-independent vascular leak is dependent on endothelial glycocalyx components. *PLOS Pathog.* **13**, e1006673 (2017).

221. Wan, S.-W. *et al.* Protection against Dengue Virus Infection in Mice by Administration of Antibodies against Modified Nonstructural Protein 1. *PLoS One* **9**, e92495 (2014).

222. Sridharan, A. *et al.* Inhibition of Megakaryocyte Development in the Bone Marrow Underlies Dengue Virus-Induced Thrombocytopenia in Humanized Mice. *J. Virol.* **87**, 11648–11658 (2013).

223. Noisakran, S. *et al.* Infection of bone marrow cells by dengue virus in vivo. *Exp. Hematol.* **40**, 250–259 (2012).

224. Clark, K. B. *et al.* Multiploid CD61+ Cells Are the Pre-Dominant Cell Lineage Infected during Acute Dengue Virus Infection in Bone Marrow. *PLoS One* **7**, e52902 (2012).

225. Lin, C. F. *et al.* Generation of IgM anti-platelet autoantibody in dengue patients. *J. Med. Virol.* **63**, 143–149 (2001).

226. Chuang, Y.-C. *et al.* Dengue Virus-Induced Autoantibodies Bind to Plasminogen and Enhance Its Activation. *J. Immunol.* **187**, 6483–6490 (2011).

227. Lin, C.-F. *et al.* Antibodies from dengue patient sera cross-react with endothelial cells and induce damage. *J. Med. Virol.* **69**, 82–90 (2003).

228. Guy, B., Briand, O., Lang, J., Saville, M. & Jackson, N. Development of the Sanofi Pasteur tetravalent dengue vaccine: One more step forward. *Vaccine* **33**, 7100–7111 (2015).

229. Poo, J. *et al.* Live-attenuated Tetravalent Dengue Vaccine in Dengue-naïve Children, Adolescents, and Adults in Mexico City. *Pediatr. Infect. Dis. J.* **30**, 9–17 (2011).

230. Capeding, R. Z. *et al.* Live-attenuated, tetravalent dengue vaccine in children, adolescents and adults in a dengue endemic country: Randomized controlled phase I trial in the Philippines. *Vaccine* **29**, 3863–3872 (2011).

231. Lanata, C. F. *et al.* Immunogenicity and safety of tetravalent dengue vaccine in 2–11 year-olds previously vaccinated against yellow fever: Randomized, controlled, phase II study in Piura, Peru. *Vaccine* **30**, 5935–5941 (2012).

232. Sin Leo, Y. *et al.* Immunogenicity and safety of recombinant tetravalent dengue vaccine (CYD-TDV) in individuals aged 2–45 years. *Hum. Vaccin. Immunother.* **8**, 1259–1271 (2012).

233. Ngoc Huu, T. *et al.* Safety and Immunogenicity of Recombinant, Live Attenuated Tetravalent Dengue Vaccine (CYD- TDV) in Healthy Vietnamese Adults and Children. *J. Vaccines Vaccin.* **03**, 1–7 (2012).

234. Villar, L. *et al.* Efficacy of a Tetravalent Dengue Vaccine in Children in Latin America. *N. Engl. J. Med.* **372**, 113–123 (2015).

235. Hadinegoro, S. R. *et al.* Efficacy and Long-Term Safety of a Dengue Vaccine in Regions of Endemic Disease. *N. Engl. J. Med.* **373**, 1195–1206 (2015).

236. Capeding, M. R. *et al.* Clinical efficacy and safety of a novel tetravalent dengue vaccine in healthy children in Asia: a phase 3, randomised, observer-masked, placebo-controlled trial. *Lancet* **384**, 1358–1365 (2014).

237. Sabchareon, A. *et al.* Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. *Lancet* **380**, 1559–1567 (2012).

238. Halstead, S. B. Dengvaxia sensitizes seronegatives to vaccine enhanced disease regardless of age. *Vaccine* **35**, 6355–6358 (2017).

239. Daep, C. A., Muñoz-Jordán, J. L. & Eugenin, E. A. Flaviviruses, an expanding threat in public health: focus on dengue, West Nile, and Japanese encephalitis virus. *J. Neurovirol.* **20**, 539–560 (2014).

240. Hills, S. L., Fischer, M. & Petersen, L. R. Epidemiology of Zika Virus Infection. *J. Infect. Dis.* **216**, 868–874 (2017).

241. Li, J. *et al.* Cross-protection induced by Japanese encephalitis vaccines against different genotypes of Dengue viruses in mice. *Sci. Rep.* **6**, 19953 (2016).

242. Guirakhoo, F. *et al.* Live attenuated chimeric yellow fever dengue type 2 (ChimeriVax-DEN2) vaccine: Phase I clinical trial for safety and immunogenicity: effect of yellow fever pre-immunity in induction of cross neutralizing antibody responses to all 4 dengue serotypes. *Hum. Vaccin.* **2**, 60–67

243. Chan, K. R. *et al.* Cross-reactive antibodies enhance live attenuated virus infection for increased immunogenicity. *Nat. Microbiol.* **1**, 16164 (2016).

244. Scott, R. M. *et al.* Dengue 2 vaccine: dose response in volunteers in relation to yellow fever immune status. *J. Infect. Dis.* **148**, 1055–1060 (1983).

245. Putvatana, R., Yoksan, S., Chayayodhin, T., Bhamarapravati, N. & Halstead, S. B. Absence of dengue 2 infection enhancement in human sera containing Japanese encephalitis antibodies. *Am. J. Trop. Med. Hyg.* **33**, 288–294 (1984).

246. Halstead, S. B., Porterfield, J. S. & O'Rourke, E. J. Enhancement of dengue virus infection in monocytes by flavivirus antisera. *Am. J. Trop. Med. Hyg.* **29**, 638–642 (1980).

247. Bardina, S. V *et al.* Enhancement of Zika virus pathogenesis by preexisting antiflavivirus immunity. *Science* **356**, 175–180 (2017).

248. Anderson, K. B. *et al.* Preexisting Japanese encephalitis virus neutralizing antibodies and increased symptomatic dengue illness in a School-Based cohort in Thailand. *PLoS Negl. Trop. Dis.* **5**, e1311 (2011).

249. Kurane, I., Brinton, M. A., Samson, A. L. & Ennis, F. A. Dengue virus-specific, human CD4+ CD8- cytotoxic T-cell clones: multiple patterns of virus cross-reactivity recognized by NS3-specific T-cell clones. *J. Virol.* **65**, 1823–1828 (1991).

250. Aihara, H., Takasaki, T., Matsutani, T., Suzuki, R. & Kurane, I. Establishment and characterization of Japanese encephalitis virus-specific, human CD4(+) T-cell clones: flavivirus cross-reactivity, protein recognition, and cytotoxic activity. *J. Virol.* **72**, 8032–8036 (1998).

251. Herrera, B. B. *et al.* Sustained Specific and Cross-Reactive T Cell Responses to Zika and Dengue Virus NS3 in West Africa. *J. Virol.* **92**, 1992–1217 (2018).

252. Reynolds, C. J. *et al.* T cell immunity to Zika virus targets immunodominant epitopes that show cross-reactivity with other Flaviviruses. *Sci. Rep.* **8**, 672 (2018).

253. Blom, K., Sandberg, J. T., Loré, K. & Ljunggren, H.-G. Prospects for induction of CD8 T cell-mediated immunity to Zika virus infection by yellow fever virus vaccination. *J. Intern. Med.* **282**, 206–208 (2017).

254. Wen, J. *et al.* Dengue virus-reactive CD8+T cells mediate cross-protection against subsequent Zika virus challenge. *Nat. Commun.* **8**, 1459 (2017).

255. Grifoni, A. *et al.* Prior Dengue virus exposure shapes T cell immunity to Zika virus in humans. *J. Virol.* **91**, 1469–1477 (2017).

256. Johnson, B. W., Russell, B. J. & Lanciotti, R. S. Serotype-Specific Detection of Dengue Viruses in a Fourplex Real-Time Reverse Transcriptase PCR Assay. *J. Clin. Microbiol.* **43**, 4977–4983 (2005).

257. Betts, M. R. *et al.* Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J. Immunol. Methods* **281**, 65–78 (2003).

258. Gatchalian, S. *et al.* Comparison of the immunogenicity and safety of measles vaccine administered alone or with live, attenuated Japanese encephalitis SA 14-14-2 vaccine in Philippine infants. *Vaccine* **26**, 2234–2241 (2008).

259. Mackenzie, J. S., Gubler, D. J. & Petersen, L. R. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat. Med.* **10**, 98–109 (2004).

260. The World Health Organization (WHO). Guidelines for plaque reduction neutralization testing of human antibodies to dengue viruses. *WHO* **Geneva**, (2003).

261. Stiasny, K., Kiermayr, S., Holzmann, H. & Heinz, F. X. Cryptic Properties of a Cluster of Dominant Flavivirus Cross-Reactive Antigenic Sites. *J. Virol.* **80**, 9557–9568 (2006).

262. Dejnirattisai, W. *et al.* Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with zika virus. *Nat. Immunol.* **17**, 1102–1108 (2016).

263. Smith, S. A. *et al.* Dengue Virus prM-Specific Human Monoclonal Antibodies with Virus Replication-Enhancing Properties Recognize a Single Immunodominant Antigenic Site. *J. Virol.* **90**, 780–789 (2016).

264. Midgley, C. M. *et al.* An in-depth analysis of original antigenic sin in dengue virus infection. *J. Virol.* **85**, 410–421 (2011).

265. Vinodkumar, C. S. *et al.* Episode of coexisting infections with multiple dengue virus serotypes in central Karnataka, India. *J. Infect. Public Health* **6**, 302–306 (2013).

266. Sasaki, S. *et al.* Limited efficacy of inactivated influenza vaccine in elderly individuals is associated with decreased production of vaccine-specific antibodies. *J. Clin. Invest.* **121**, 3109–3119 (2011).

267. Ginsburg, A. S., Meghani, A., Halstead, S. B. & Yaich, M. Use of the live attenuated Japanese Encephalitis vaccine SA 14–14–2 in children: A review of safety and tolerability studies. *Hum. Vaccin. Immunother.* **13**, 2222–2231 (2017).

268. Kumar, R., Tripathi, P. & Rizvi, A. Effectiveness of One Dose of SA 14-14-2 Vaccine against Japanese Encephalitis. *N. Engl. J. Med.* **360**, 1465–1466 (2009).

269. Khan, S. A. *et al.* Immunogenicity & safety of a single dose of live-attenuated Japanese encephalitis vaccine SA 14-14-2 in adults. *Indian J. Med. Res.* **144**, 886–892 (2016).

270. Mutheneni, S. R., Morse, A. P., Caminade, C. & Upadhyayula, S. M. Dengue burden in India: recent trends and importance of climatic parameters. *Emerg. Microbes Infect.* **6**, 70–78 (2017).

271. Tsai, W.-Y. *et al.* Complexity of Neutralizing Antibodies against Multiple Dengue Virus Serotypes after Heterotypic Immunization and Secondary Infection Revealed by

In-Depth Analysis of Cross-Reactive Antibodies. *J. Virol.* **89**, 7348–7362 (2015).

272. O'Donnell, C. D. *et al.* Humans and Ferrets with Prior H1N1 Influenza Virus Infections Do Not Exhibit Evidence of Original Antigenic Sin after Infection or Vaccination with the 2009 Pandemic H1N1 Influenza Virus. *Clin. Vaccine Immunol.* **21**, 737–746 (2014).

273. Choi, Y. S. *et al.* Reduced antibody responses to the pandemic (H1N1) 2009 vaccine after recent seasonal influenza vaccination. *Clin. Vaccine Immunol.* **18**, 1519–1523 (2011).

274. Sohn, Y. M. *et al.* Primary and booster immune responses to SA14-14-2 Japanese encephalitis vaccine in Korean infants. *Vaccine* **17**, 2259–2264 (1999).

275. Halstead, S. B. & Russell, P. K. Protective and immunological behavior of chimeric yellow fever dengue vaccine. *Vaccine* **34**, 1643–1647 (2016).

276. Terzian, A. C. B. *et al.* Viral Load and Cytokine Response Profile Does Not Support Antibody-Dependent Enhancement in Dengue-Primed Zika Virus–Infected Patients. *Clin. Infect. Dis.* **65**, 1260–1265 (2017).

277. Pantoja, P. *et al.* Zika virus pathogenesis in rhesus macaques is unaffected by pre-existing immunity to dengue virus. *Nat. Commun.* **8**, 15674 (2017).

278. Gordon, A. *et al.* Prior dengue virus infection and risk of Zika: A pediatric cohort in Nicaragua. *PLoS Med.* **16**, e1002726 (2019).

279. Pedroso, C. *et al.* Cross-Protection of Dengue Virus Infection against Congenital Zika Syndrome, Northeastern Brazil. *Emerg. Infect. Dis.* **25**, 1485–1493 (2019).

280. Kurane, I., Hebblewaite, D., Brandt, W. E. & Ennis, F. A. Lysis of dengue virus-infected cells by natural cell-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. *J. Virol.* **52**, 223–230 (1984).

281. García, G. *et al.* Antibodies from patients with dengue viral infection mediate cellular cytotoxicity. *J. Clin. Virol.* **37**, 53–57 (2006).

282. Cardosa, M., Porterfield, J. & Gordon, S. Complement Receptor Mediates Enhanced Flavivirus Replication in Macrophages. *J. Exp. Med.* **158**, 258–263 (1983).

283. Mehlhop, E., Ansarah-Sobrinho, C., Johnson, S., Engle, M., Fremont, D.H., Pierson, T.C., Diamond, M. S. Complement Protein C1q Inhibits Antibody-Dependent Enhancement of Flavivirus Infection in an IgG Subclass-Specific Manner. *Cell Host Microbe* **2**, 417–426 (2007).

284. Markoff, L. Points to consider in the development of a surrogate for efficacy of novel Japanese encephalitis virus vaccines. *Vaccine* **18**, 26–32 (2000).

285. Larena, M., Regner, M. & Lobigs, M. Cytolytic effector pathways and IFN-γ help protect against Japanese encephalitis. *Eur. J. Immunol.* **43**, 1789–1798 (2013).

286. Akondy, R. S. *et al.* The yellow fever virus vaccine induces a broad and polyfunctional human memory CD8+ T cell response. *J. Immunol.* **183**, 7919–7930 (2009).

287. James, E. A. *et al.* Yellow fever vaccination elicits broad functional CD4+ T cell responses that recognize structural and nonstructural proteins. *J. Virol.* **87**, 12794–12804 (2013).

288. Chu, H., George, S. L., Stinchcomb, D. T., Osorio, J. E. & Partidos, C. D. CD8+ T-cell Responses in Flavivirus-Naive Individuals Following Immunization with a Live-Attenuated Tetravalent Dengue Vaccine Candidate. *J. Infect. Dis.* **212**, 1618–1628 (2015).

289. Angelo, M. A. *et al.* Human CD4+T Cell Responses to an Attenuated Tetravalent Dengue Vaccine Parallel Those Induced by Natural Infection in Magnitude, HLA Restriction, and Antigen Specificity. *J. Virol.* **91**, 2157–2168 (2017).

290. Weiskopf, D. *et al.* The Human CD8+ T Cell Responses Induced by a Live Attenuated Tetravalent Dengue Vaccine Are Directed against Highly Conserved Epitopes. *J. Virol.* **89**, 120–128 (2015).

291. Weiskopf, D. *et al.* Immunodominance Changes as a Function of the Infecting Dengue Virus Serotype and Primary versus Secondary Infection. *J. Virol.* **88**, 11383–11394 (2014).

292. Rivino, L. *et al.* Differential Targeting of Viral Components by CD4+ versus CD8+ T Lymphocytes in Dengue Virus Infection. *J. Virol.* **87**, 2693–2706 (2013).

293. Tian, Y., Grifoni, A., Sette, A. & Weiskopf, D. Human T Cell Response to Dengue Virus Infection. *Front. Immunol.* **10**, 2125 (2019).

294. Santos, R. *et al.* Improvement of IFNg ELISPOT Performance Following Overnight Resting of Frozen PBMC Samples Confirmed Through Rigorous Statistical Analysis. *Cells* **4**, 1–18 (2014).

295. Yang, K. D., Yeh, W.-T., Yang, M.-Y., Chen, R.-F. & Shaio, M.-F. Antibody-dependent enhancement of heterotypic dengue infections involved in suppression of IFNgamma production. *J. Med. Virol.* **63**, 150–157 (2001).

296. Wen, J. *et al.* Identification of Zika virus epitopes reveals immunodominant and protective roles for dengue virus cross-reactive CD8+ T cells. *Nat. Microbiol.* **2**, 17036 (2017).

297. Owen, R. E. *et al.* Loss of T cell responses following long-term cryopreservation. *J. Immunol. Methods* **326**, 93–115 (2007).

298. Kurane, I., Zeng, L., Brinton, M. A. & Ennis, F. A. Definition of an Epitope on NS3 Recognized by Human CD4+Cytotoxic T Lymphocyte Clones Cross-Reactive for Dengue Virus Types 2, 3, and 4. *Virology* **240**, 169–174 (1998).

299. Zivna, I. *et al.* T cell responses to an HLA-B*07-restricted epitope on the dengue NS3 protein correlate with disease severity. *J. Immunol.* **168**, 5959–5965 (2002).

300. Parsons, R. *et al.* The memory T cell response to West Nile virus in symptomatic humans following natural infection is not influenced by age and is dominated by a restricted set of CD8+ T cell epitopes. *J. Immunol.* **181**, 1563–1572 (2008).

301. Zivny, J. *et al.* Partial agonist effect influences the CTL response to a heterologous dengue virus serotype. *J. Immunol.* **163**, 2754–2760 (1999).